The role of Stl1p in glycerol accumulation in osmotically stressed Icewine yeast *Saccharomyces cerevisiae* K1V1116

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ABSTRACT

The high sugar concentration in Icewine juice exerts hyperosmotic stress in the wine yeast causing water loss and cell shrinkage. To counteract the dehydration, yeast synthesize and accumulate glycerol as an internal osmolyte. In a laboratory strain of *S. cerevisiae*, *STL1* encodes for Stl1p, an H^+/glycerol symporter that is glucose inactivated, but induced upon hyperosmotic stress.

STL1, was found to be a highly upregulated gene in Icewine fermenting cells and its expression was 25-fold greater than in yeast cells fermenting diluted Icewine juice, making it one of the most differentially expressed genes between the two fermentation conditions. In addition, Icewine fermenting cells showed a two-fold higher glycerol production in the wine compared to yeast fermenting diluted Icewine juice.

We proposed that Stl1p is (1) active during Icewine fermentation and is not glucose inactivated and (2) its activity contributes to the limited cell growth observed during Icewine fermentation as a result of the dissipation of the plasma membrane proton gradient.

To measure the contribution of Stl1p in active glycerol transport (energy dependent) during Icewine fermentation, we first developed an Stl1p-dependent [^{14}C]glycerol uptake assay using a laboratory strain of *S. cerevisiae* (BY4742 and *ΔSTL1*) that was dependent on the plasma membrane proton gradient and therefore energy-dependent. Wine yeast K1-V1116 was also shown to have this energy dependent glycerol uptake induced under salt stress.

The expression of *STL1* and Stl1p activity were compared between yeast cells harvested from Icewine and diluted Icewine fermentations. Northern blot analysis revealed that *STL1* was expressed in cells fermenting Icewine juice but not expressed under the diluted juice conditions. Glycerol uptake by cells fermenting Icewine juice was not significantly different than cells fermenting diluted Icewine juice on day 4 and day 7 of Vidal and Riesling fermentations respectively, despite encountering greater hyperosmotic stress. Furthermore, energy- dependent glycerol uptake was not detected under either fermentation conditions.

Because our findings show that active glycerol uptake was not detected in yeast cells harvested from Icewine fermentation, it is likely that Stl1p was glucose inactivated despite the hyperosmotic stress induced by the Icewine juice and therefore did not play a role in active glycerol uptake during Icewine fermentation.

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I would like to thank my supervisor Dr. Debbie Inglis who showed me great support during the completion of this project. Thank you for opening the doors of your lab and allowing me to explore my research skills. I would also like to thank the committee members of my project, Dr. Atkinson and Dr. Després for reviewing my thesis and for their insightful comments. Great thanks for all of my friends in IH 210 lab: Ailin, Stephanie, Fei and Eric. We shared great moments together. Special thanks to Dr. Ailin Beh and Dr. Stephanie Martin who have been a great support for me. I will always appreciate you. A big thanks to Matilda and Wendy who shared our lab and were always smiling. Last thanks is to the CCOVI team.

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ABBREVIATION LIST

STL1- Sugar transport like

HOG MAPK- high osmolarity glycerol mitogen activated protein kinase

CCCP- carbonylcyanide-*m*-chlorophenylhydrazone

GPD- glyceraldehyde-3-phosphate dehydrogenase

GPP- glycerol-3-phosphat phosphatase

S. cerevisiae- Saccharomyces cerevisiae

SGD- *Saccharomyces cerevisiae* database

1 INTRODUCTION

1.1 INTRODUCTION TO THE PROBLEM

Production of glycerol by yeast in response to hyperosmotic stress is an important adaptation mechanism during Icewine fermentation. Glycerol is an important osmolyte that allows yeast cells to adapt to the hyperosmotic stress. Glycerol concentration has been reported to be 4.85 g/L in Riesling Icewine juice and 1.9 g/L in Vidal Icewine juice (Pigeau and Inglis, 2005; Pigeau and Inglis, 2007; Pigeua, et al., 2007; Martin, 2008). In addition, wine yeast cells synthesize glycerol during Icewine fermentation to act as an internal osmolyte. Surprisingly, the cells release the glycerol early in the fermentation (Pigeau and Inglis, 2005; Martin, 2008).

Interestingly, *STL1* encoding Stl1p, an active glycerol transporter (energy dependent), was found to be a highly upregulated gene in Icewine fermenting cells and its expression was 25-fold greater than in yeast cells fermenting diluted Icewine juice, making it one of the most differentially expressed genes between the two fermentation conditions (Martin, 2008).

STL1 encodes for the sugar-like transport protein (Stl1p) and was recently characterized as an H^+/glycerol membrane symporter in laboratory yeast strains (Ferreria et al., 2005). It is one of the most highly induced genes during the response to hyperosmotic stress (Rep et al., 2000; Posas and Saito, 1997; Yale and Bohnert, 2001). Under non-hyperosmotic stress conditions St1lp is glucose inactivated,

however this inactivation is overcome upon exposure to saline hyperosmotic stress (Ferreria et al., 2005).

Because *STL1* expression is a dynamic process that depends on the yeast strain and the environmental conditions the cells are exposed to, it is possible that Stl1p is induced in Icewine fermenting cells to actively uptake glycerol from the fermenting media. If this is true, increased active transport of glycerol into the yeast cell by Stl1p may lead to the dissipation of the proton gradient across the plasma membrane and thus eliminate the driving force of nutrient entry into the cell (reviewed by Boulton et al., 1998; Horák, 1986). These events may direct ATP utilization within the yeast cell toward reestablishment of the proton gradient, rather than cell growth. Thus, the limited cell growth observed during Icewine fermentation may be a result of reduced nutrient uptake due to high induction of Stl1p.

Analysis of Icewine juices from the Niagara Region of Ontario, Canada shows an average assimilable nitrogen content of 555 mg N/L for 297 Vidal Icewine juices and 461 mg N/L for 24 Riesling Icewine juices (Ziraldo and Kaiser, 2007). Even though a sufficient amount of nitrogen is present in the Icewine juice, still nitrogen uptake is reduced during Icewine fermentation (Martin, 2008)

In previous studies, wine yeast only used 112 mg /L of nitrogen during Icewine fermentation, even though 389 mg/L was available in the initial juice (Pigeau and Inglis, 2005). Despite the fact that nitrogen and sugar concentrations are not limiting in Icewine fermentation, cells were only reported to double 2-3 times

during fermentation as opposed to 7-8 times during a table wine fermentation (Kontkanen et al., 2004).

The role that Stl1p plays in wine yeast during Icewine fermentation is still unknown. Thus, the goal of this research is to characterize the function of Stl1p in hyperosmotically stressed Icewine fermenting cells and determine if Stl1p actively uptakes glycerol into the cell during Icewine fermentation.

If Stl1p indeed contributes to the limited cell growth observed during Icewine fermentation, the induction of this protein in wine yeast may not be desirable given that it slows down the fermentation process. However, active Stl1p may provide an advantage to wine yeast fermenting Icewine if it is found to be active during fermentation and assisting the yeast to overcome the stress of the environment. Since there is no commercial yeast strain that has been specifically selected for Icewine fermentation in the wine industry, the expression of *STL1* could be used as a biomarker for strain screening purposes depending on its contribution to wine yeast during Icewine fermentation. The findings of this project may assist in this selection process for strains that may potentially ferment Icewine juice with improved efficiency.

1.2 OBJECTIVES

The goal of this project was to investigate if Stl1p H+/glycerol symporter actively transports glycerol into wine yeast cells in response to hyperosmotic stress induced by Icewine fermentation. To pursue this goal, first it was necessary to design a glycerol uptake assay that measures energy dependent glycerol transport specific for Stl1p, often referred to in this thesis as "Stl1p-dependent active glycerol uptake" and second, to use this assay to compare Stl1p-dependent glycerol uptake in wine yeast fermenting Icewine and diluted Icewine juice.

1.3 EXPERIMENTAL DESIGN

To investigate if Stl1p H+/glycerol symporter actively uptakes glycerol into wine yeast during Icewine fermentation, it was necessary to design a glycerol uptake assay specific for Stl1p. For this purpose, two laboratory *S. cerevisiae* strains were used: the laboratory mutant strain *ΔSTL1* where the *STL1* gene is not expressed and its parental strain BY4742. Both strains were subjected to salt stress and *STL1* induction was confirmed in the parent strain using Northern blot analysis. Under the same induction conditions, cells of both parent and mutant yeast strains were harvested to measure glycerol uptake in response to hyperosmotic stress. To follow the level of glycerol uptake and accumulation in the cell, radiolabeled glycerol ([^{14}C]glycerol) was used.

In order to determine the optimal ([^{14}C]glycerol assay conditions that show specificity for Stl1p activity, [^{14}C]glycerol uptake was compared under three different glycerol concentrations (4 , 10 and 65 mM). The low glycerol concentrations are published concentratios under which Stl1p activity was measured (Ferreria, et al., 2005). The 65 mM glycerol concentration was chosen to determine if Stl1p activity is measurable using the glycerol assay in the presence of higher glycerol concentration. Martin (2008) showed that at day 4 of Icewine fermentation, the glycerol concentration in the Icewine juice reached 65 mM, and at the same timepoint, *STL1* expression peaked.

To determine if glycerol uptake depends on the presence of Stl1p, glycerol initial uptake rates were compared between *ΔSTL1* and its parent strain. To investigate if

Stl1p glycerol uptake is energy dependent, glycerol uptake was measured in the presence of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP). This uncharged lipid-soluble weak acid is able to cross the yeast membrane and release a proton in the cytosol, thus leading to the dissipation of the proton gradient across the plasma membrane (reviewed in Spencer and Spencer, 1997; Figure 1.3.1 C). If glycerol transport by the Stl1p depends on the proton motive force, then in the presence of CCCP, Stl1p-dependent glycerol uptake should be inhibited in the parent strain. To investigate if wine yeast displays Stl1p-dependent glycerol uptake, cells were subjected to the same saline stress conditions in order to induce Stl1p and harvested to measure glycerol accumulation in the presence and absence of CCCP (Figure 1.3.1 A).

Once the optimal assay conditions were determined to show Stl1p-dependent active glycerol uptake in wine yeast, Stl1p activity was compared between yeast cells fermenting Icewine and cells fermenting diluted Icewine juice in the presence and absence of the protonophore (Figure 1.3.1 B).

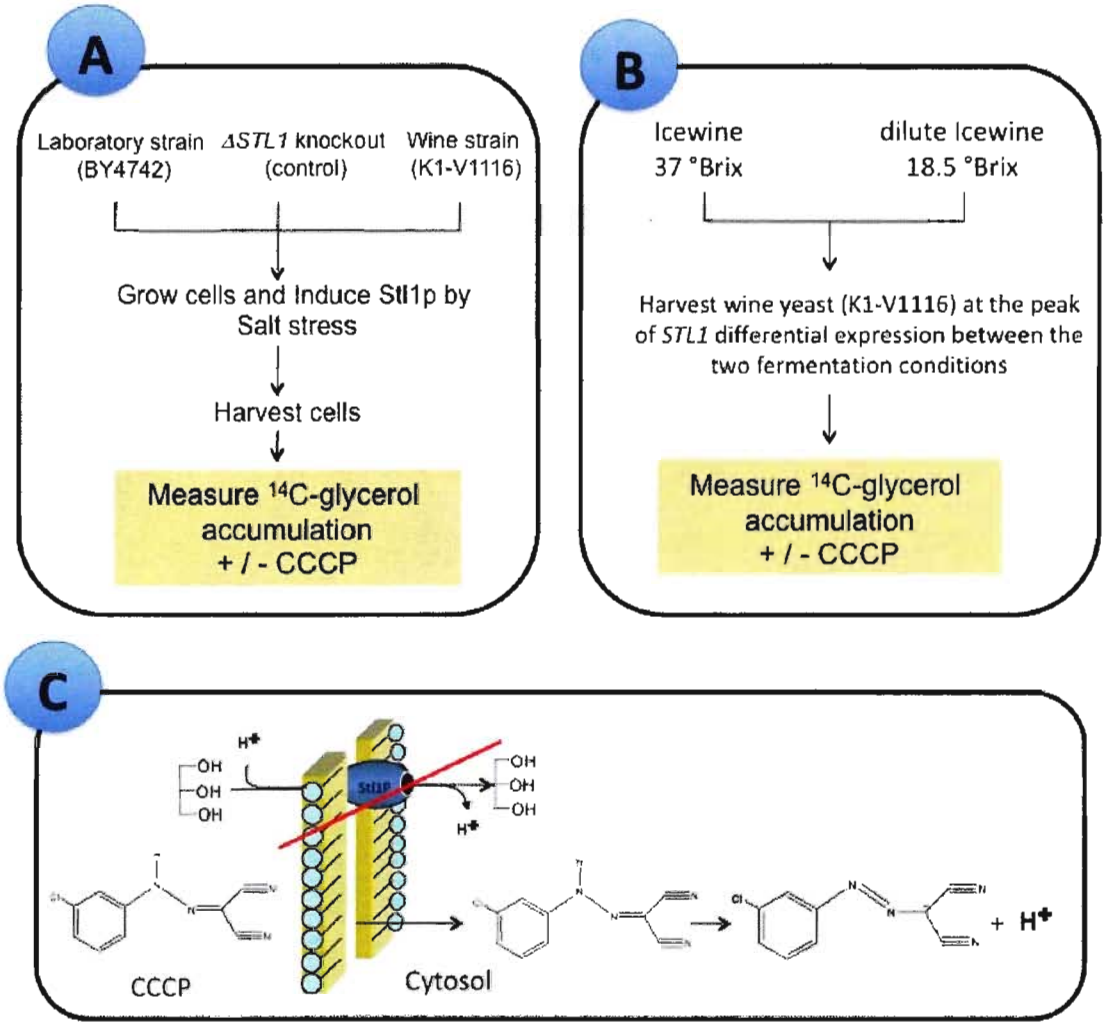

Figure 1.3.1: Experimental design of [^{14}C]glycerol uptake assays. (A) [^{14}C]glycerol uptake by BY4742, *ΔSTL1* knockout and wine yeast K1-V1116 was measured upon exposure to saline stress in the presence and absence of CCCP. (B) Stl1p-dependent [^{14}C]glycerol uptake was measured in wine yeast during Icewine and dilute Icewine fermentations in the presence and absence of CCCP.(C) CCCP as indicator for glycerol active uptake. CCCP dissipates the yeast plasma membrane proton gradient by crossing the membrane and releasing a proton in the cytosol, thus inhibiting active glycerol uptake via Stl1p.

2 LITERATURE REVIEW

2.1 Icewine: Production and challenges

Icewine (Eiswein in German) is a sweet dessert wine made from the juice of naturally frozen grapes. In Canada, the wine is named Icewine only if the grapes are picked and immediately pressed at temperatures lower than -8°C (VQA, 1999). During the pressing, water ice crystals are trapped in the grape and therefore not transferred to the extracted juice. The substantial reduction in water content results in juice that is highly concentrated with soluble solids such as sugars, acids and nitrogenous compounds. The concentration of soluble solids in Canadian Icewine juice commonly falls within the range of 38°Brix to 42°Brix with a minimum allowed limit of 35°Brix (VQA, 1999).

Canada is the world's largest producer of Icewine, with the majority of production originating from the Niagara Peninsula region in the southern portion of the province of Ontario. In 2007, it was reported that Ontario produced approximately 1.25 million liters, an increase of 123% in volume produced from the previous year (VQA, 2008).

Icewine producers face different challenges throughout the production process of Icewine. Annual yields of Icewine may vary each year depending on the weather conditions, a factor which eventually determines the time point of grape harvest. The longer the grapes are left on the vine, the more they are susceptible to damage by bird feeding on the crop and continued dehydration (VQA, 2008). More challenges are introduced during the fermentation process. Because of the

concentrated nature of the Icewine juice, fermentations are often sluggish, taking months to reach the desired ethanol levels (10% v/v) and usually end with high levels of residual sugar with an average of 214.7 g L^{-1} (Nurgel et al., 2004).

The complex composition of the Icewine must is the major contributor to the lengthy fermentation and the lower levels of ethanol. Wine yeast fermenting Icewine juice experiences hyperosmotic stress induced by the high concentration of soluble solids found in the must. Once inoculated to the hypertonic environment of the Icewine must, yeast cells loose water content due to osmosis and therefore shrink. Within minutes yeast cells activate an initial stress response to counteract the dehydration effect of the hyperosmotic stress. Within hours, yeast cells activate the high osmolarity glycerol (HOG) pathway, a signaling pathway that eventually leads to the upregulation of glycerol synthesis genes (Erasmus et al., 2003). Following the initial response, cells turn on an adaptive response that requires changes in sugar metabolism. Cells fermenting Icewine experience reduced growth rates and therefore only double 2-3 times throughout the course of the fermentation in comparison to table wine where cells double at least 7-8 times (Pigeau and Inglis, 2005). Studies show that during Icewine fermentation, a higher portion of sugar energy is directed towards the production of glycerol and acetic acid rather than cell growth (Pigeau and Inglis, 2005; Pigeau et al., 2007; Martin, 2008).

The production and accumulation of glycerol during the yeast response to hyperosmotic stress is an important adaptation mechanism against the dehydration effect of Icewine juice on wine yeast. The accumulation of glycerol is necessary for

the continuation of biochemical processes in the cell, even when water content in the cell is low, and thereby allowing the cell to survive (Reviewed by Hohmann et al., 2007). A survey of 50 Canadian Icewines reported that the average concentration of glycerol in Canadian Icewines is 12.4 g L^{-1}, where as reported values of table wine ranges from 1.4 to 10.6 g L^{-1} (Nurgel et al., 2004).

Commercial yeast strains were selectively chosen to ferment wine due to their tolerance to different stresses introduced during fermentation. It is possible that some of these strains are better suited to ferment Icewine juice with greater efficiency, however no biomarkers have been identified to select for these strains. It is essential to investigate the role of Stl1p in commercial wine yeast strains during Icewine fermentation to better understand yeast adaptation mechanisms and perhaps use *STL1* as a biomarker to select for strains that can manage Icewine fermentation processes more efficiently and consequently increase Icewine quality.

2.2 *Yeast response to hyperosmotic stress*

An increase in environmental osmolarity evokes different response mechanisms in *S. cerevisiae* to adapt to the hyperosmotic stress. This response has been extensively studied in laboratory yeast strains of *S. cerevisiae* (reviewed by Hohmann , 2009). Due to excessive water efflux, yeast lose membrane turgor pressure and therefore the cells shrink. As a result, cells will increase glycerol retention due to its role as an internal osmolyte. Within minutes, the glycerol diffusion rate decreases in yeast cells and they shut down the glycerol efflux channel Fps1p to prevent glycerol loss from the cell (Figure 2.2.1.a and .b, Tamás et al., 1999).

At the same time yeast activates the high osmolarity glycerol (HOG) pathway (Figure 2.2.1.c) (reviewed by Hohmann, 2009). This MAP kinase mediated signaling transduction pathway controls the expression of many stress responsive genes, among them *GPD1*, to control glycerol synthesis and *STL1*, to control glycerol uptake (Figure 2.2.1.c, Ferreria et al., 2005). After approximately 15 minutes, cells initiate energy dependent uptake of extracellular glycerol through the induction of Stl1p, an H+/glycerol symporter (Ferreria et al., 2005). All of these events lead to the cells adaptation and survival.

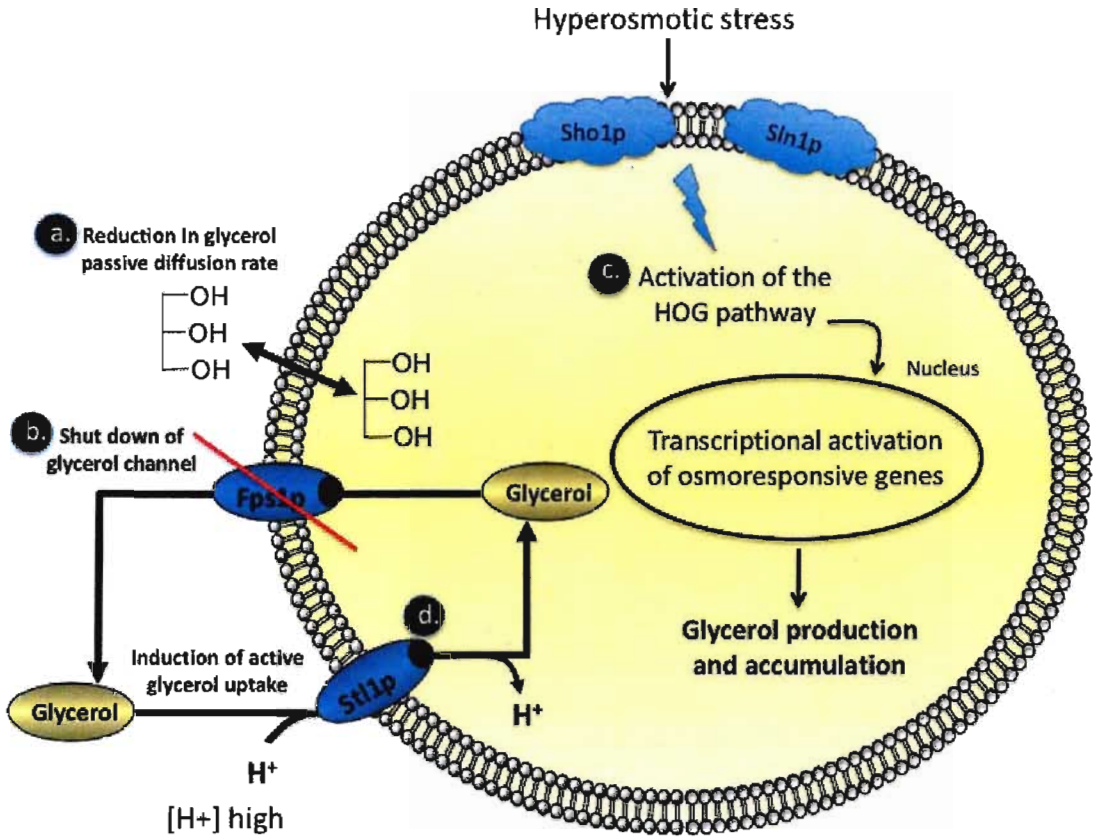

Figure 2.2.1: Yeast response to hyperosmotic stress in *S. cerevisiae*. Hyperosmotic stress results in (a) the reduction of glycerol passive diffusion out of the cell, (b) shut down of Fps1p glycerol channel and (c) activation of the high osmolarity glycerol (HOG) pathway to upregulate the expression of glycerol synthesis genes. At the same time, yeast induces active glycerol uptake of Stl1p (d). (Hohmann, 2009).

2.2.1 The effect of changes in environment osmolarity on yeast turgor pressure and water potential

The semipermeable membrane of yeast allows water molecules to freely flow into or out of the cell in order to reach water equilibrium with the environment (reviewed by Blomberg and Adler, 1992). Besides passive diffusion, water can also cross the membrane via facilitated diffusion mediated by different aquaporins (Pettersson et al., 2005) which act as regulators for water movement across the plasma membrane. This water flow phenomena, named osmosis, directly depends on the solute concentrations the cells are exposed to. Since active cellular processes occur in aqueous solutions, changes in the cellular osmotic balance must trigger changes in cell physiology. These changes are critical for cell survival and growth in the new environmental conditions.

Water will always flow across the semi-permeable membrane towards the higher solute concentration, where the potential of water is lower (Hohmann and Mager, 2003). At hypertonic conditions (low water potential in environment), where the concentration of solutes in the surrounding media is higher than the intracellular concentration, water flows out of the yeast cell to reach thermodynamic water equilibrium. As a result, the cells dehydrate and shrink (Figure 2.2.1.1). Conversely, at hypotonic conditions (high water potential in environment), when the concentration of solutes in the outside environment is lower than the intracellular concentration, water flows into the cell and therefore the cells swell.

Water potential not only depends on the concentration of solutes (osmotic potential) in the cell but also on the turgor pressure of the cell (Blomberg and Adler, 1992). Under non-stressed conditions, yeast cells keep a slightly higher internal osmotic pressure than that of the surrounding medium. The pressure difference is counteracted by cell wall resistance and is referred to as cell turgor pressure (Hohmann and Mager, 2003).

Hyperosmotic stress causes yeast to lose water content and therefore turgor pressure. These events eventually lead to structural changes in the membrane of *S. cerevisiae*. In response to these changes, the yeast activates different mechanisms that lead to the synthesis, accumulation and retention of glycerol, an important compatible solute that allows the yeast to adapt and survive the effects of hyperosmotic stress. These mechanisms are discussed in detail in sections 2.2.2.3 and 2.2.2.4.

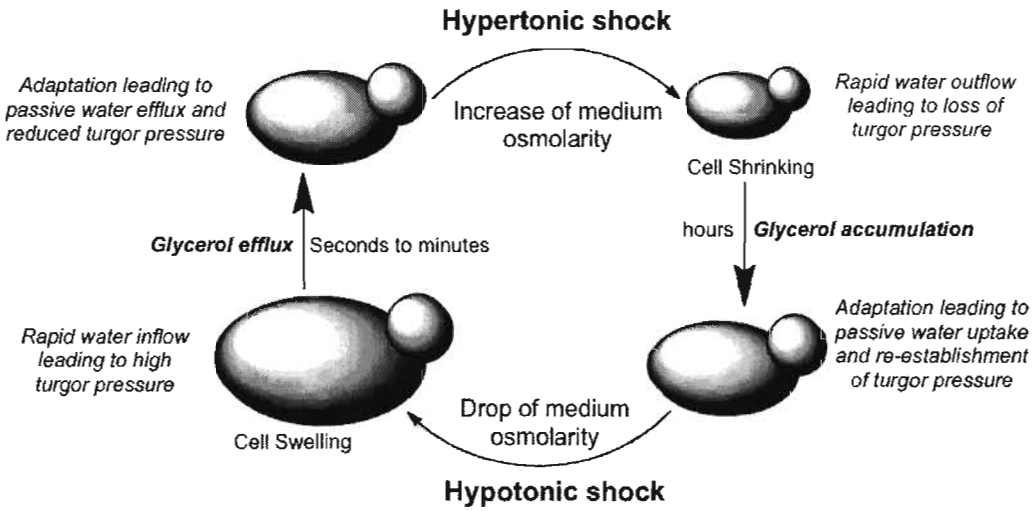

Figure 2.2.1.1: A schematic representation of the effect of changes in environmental osmolarity on budding yeast size and turgor pressure.

2.2.2 The role of glycerol as an internal osmolyte in *S. cerevisiae*

In order to understand yeast response to hyperosmotic stress, it is necessary to investigate the role glycerol plays as an internal osmolyte when yeast is exposed to hypertonic environments such as Icewine juice.

A general mechanism by which microorganisms counteract the dehydration effects of hyperosmotic stress is through the uptake and production of one or more specific solutes called osmolytes or osmoprotectants (Yancey et al., 1982).

Osmolytes are accumulated in microorganisms in order to control and balance water levels in the cell. These solutes are also referred to as compatible solutes because cells can accumulate osmolytes in high concentration without giving rise to appreciable enzyme inhibition or inactivation, thus allowing cellular processes to continue even though water availability is low in the cell. Osmolytes can be accumulated in a microorganism either through synthesis or through uptake from the surrounding medium (Pollard and Wyn Jones, 1979).

Glycerol is the main compatible solute of fungi among other polyhydroxy alcohols such as arabinitol and erythritol (Blomberg and Adler, 1992) and the sole compatible solute in *S. cerevisiae* during response to hyperosmotic stress (Hohmann and Mager, 2003).

What is the role that glycerol plays during response to hyperosmotic stress? Glycerol is accumulated in the yeast cell to increase the internal osmolarity and induce water flow back into the shrunken cells. The increase in water content

reestablishes turgor pressure, which is required for normal membrane function (Hohmann and Mager, 2003; Blomberg and Adler, 1992).

2.2.2.1 Glycerol synthesis during hyperosmotic stress

Blomberg and Adler (1989) demonstrated that *S. cerevisiae* cells accumulated higher levels of glycerol with increased saline osmolarity (up to 1.4 M NaCl) and that increase was directly related to enhanced activity of the enzyme responsible for glycerol synthesis under hyperosmotic stress, Gpd1p. In addition, *GPD1Δ* mutants were shown to produce less than half of the glycerol produced by their wild type counterparts, and were shown to be sensitive to high saline osmolarity (Ansell et al., 1997; Albertyn et al., 1994). This evidence suggests that glycerol is an important metabolite for yeast to survive hypertonic environments, and therefore yeast induce specific glycerol synthesis genes to increase intracellular levels of glycerol to survive.

2.2.2.2 Glycerol synthesis pathways under different environmental conditions

In *S. cerevisiae,* glycerol is synthesized from the glycolytic intermediate dihydroxyacetone phosphate in two steps (Figure 2.2.2.2.1): Reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase and dephosphorylation of glycerol-3-phosphate to yield glycerol by glycerol-3-phosphate phosphatase. The reduction of DHAP to glycerol-3-phosphate is the rate-limiting step in osmotically induced glycerol formation (Remize, et al. 2003). It is catalyzed by two NAD-dependent enzymes encoded by two isogenes: *GPD1* and *GPD2*. The hydrolysis of glycerol-3-phosphate to glycerol is catalyzed by two phosphatases encoded by two isogenes: *GPP1* and *GPP2* (Larsson et al., 1993; Albertyn et al., 1994; Ansell et al., 1997; Pahlman et al., 2001). Each of these dehydrogenases and phosphatases are differentially expressed depending on the metabolic state of the cell.

The expression of *GPD1* is stimulated under hyperosmotic stress and considered to be the major isoform responsible for glycerol production during hyperosmotic stress (Larsson et al., 1993; Albertyn et al., 1994). Contrary to *GPD1*, the expression of *GPD2* is not upregulated in response to hyperosmotic stress and its mRNA levels actually drops (Ansell et al., 1997). *GPD2* is expressed in yeast for the purpose of maintaining intracellular redox balance for the NAD^+/NADH co-factor system. Even though only *GPD1* was found to be the isoform responsible for glycerol production in response to hyperosmotic stress, mutations in either *GPD1* or *GPD2* results in

sensitivity to hyperosmotic stress, indicating that both isoforms are required for the yeast to survive a hypertonic environment.

The second reaction of glycerol synthesis involves two phosphatases encoded by *GPP1* and *GPP2*. The expression of both isoforms is induced under hyperosmotic stress conditions, however, *GPP2* is expressed to a greater degree than *GPP1* (Påhlman et al., 2001; Hirayarna et al., 1995; Norbeck et al., 1996).

It appears that the Gpd1p-Gpp2p pair forms the major pathway for glycerol production in *S. cerevisiae* during response to hyperosmotic stress under aerobic conditions.

When yeast is grown under anaerobic and hyperosmotic stress conditions, the production of glycerol is required not only to increase intracellular glycerol concentration but also to maintain redox balance. The reoxidization of nicotinamide adenine dinucleotide (NADH) during glycerol synthesis allows the cell to balance the redox potential and inorganic phosphate recycling (Ansell et al., 1997). Unlike aerobic conditions where Gpd1p-Gpp2p forms the major pathway for glycerol production in *S. cerevisiae* (Påhlman et al., 2001), under anaerobic conditions glycerol production is prominently controlled by the Gpd2p-Gpp1p isoforms (Ansell et al., 1997).

During wine fermentation, wine yeast is exposed to osmotic stress under anaerobic conditions. In contrast to previous findings where anaerobic conditions induced the expression of *GPD2,* in wine fermentation *GPD1* plays a major role in glycerol formation, specifically during the first few hours of exposure to high sugar

concentration (Remize et al., 2003). Similarly, Pigeau and Inglis (2005) showed that the elevated production of glycerol in Icewine fermenting yeast cells corresponds to an increase in expression of *GPD1* but not *GPD2*. Therefore, during Icewine fermentation glycerol production appears to be mainly controlled by Gpd1p-Gpp2p isoforms (Martin, 2008; Pigeau and Inglis, 2007).

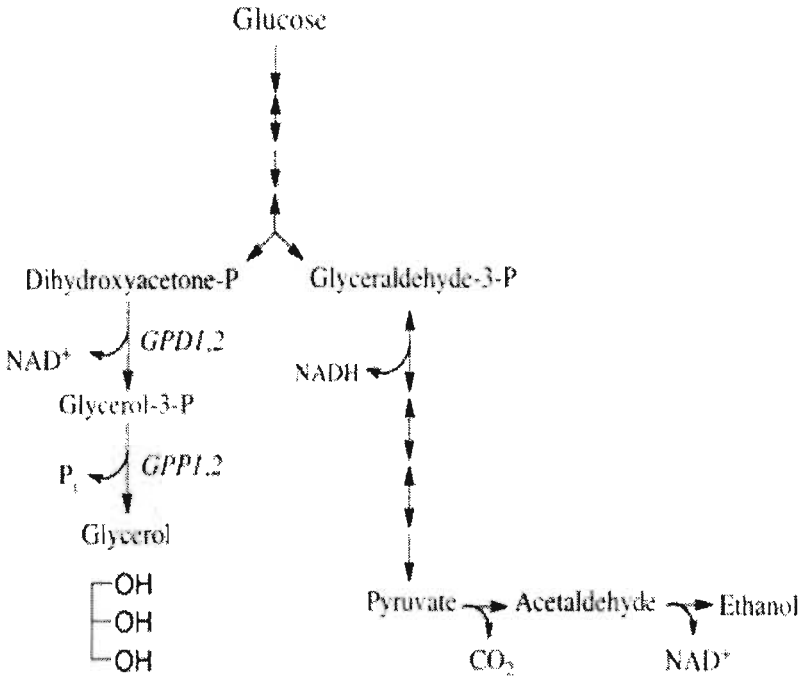

Figure 2.2.2.2.1: Glycerol synthesis and ethanol production pathway during alcoholic fermentation. Glucose is oxidized through glycolysis into two intermediates, glycerol-3-phosphate that is further oxidized to ethanol and dihydroxyacetone phosphate that is eventually converted to glycerol. Glycerol synthesis from DHAP is a two-step process that involves two pair of enzymes: Glycerol-3-phosphate dehydrogenase 1 or 2 (encoded by *GPD1* or *GPD2*) and glycerol-3-phosphatase 1 or 2 (encoded by *GPP1* or *GPP2*). (Nevoigt and Stahl,

2.2.2.3 *Activation of the HOG pathway for glycerol synthesis*

The high osmolarity glycerol (HOG) mitogen activated protein kinase (MAPK) pathway mediates the immediate transcriptional response to hyperosmotic stress in *S. cerevisiae* to allow cell adaptation and survival under high osmolarity conditions. The architecture of the MAPK signaling pathway is conserved in eukaryotes and is based on the sequential phosphorylation of three levels of mitogen activated protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) (Figure 2.2.2.3.1, reviewed in Hohmann, 2007).

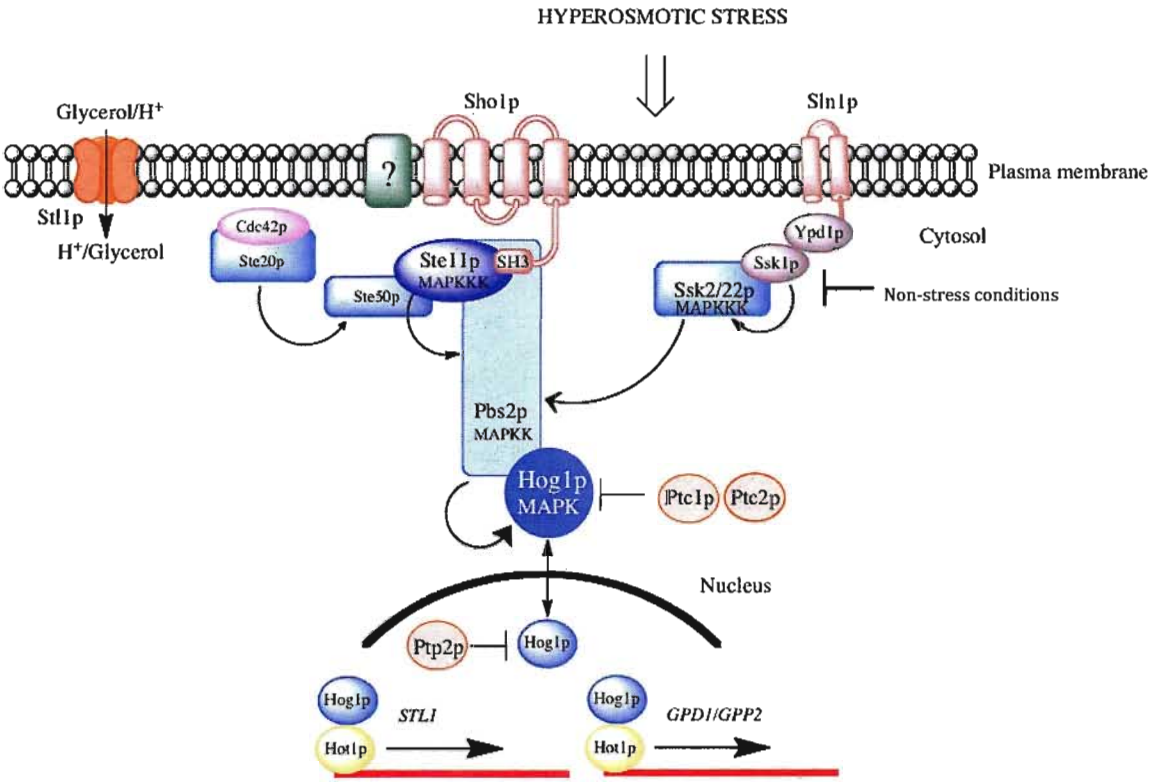

Figure 2.2.2.3.1: The HOG pathway in *S. cerevisiae* controls glycerol homeostasis. The activation of Hot1p transcription factor initiates the transcription of osmotolerant related gene, GPD1/GPP2 that control glycerol synthesis and STL1 that is responsible for glycerol uptake. (Hohmann, 2009).

The MAPKs are important signal transduction protein kinases that are involved in many facets of cellular regulation such as cell proliferation, cell differentiation, cell movement and cell death.

The presence of the appropriate stimuli will induce the phosphorylation of the MAPKKK which phosphorylates the MAPKK on its serine and threonine residues and that in turn phosphorylates the threonine/serine and tyrosine residues of the terminal kinase, the MAPK. The latter is translocated into the nucleus to activate gene expression in response to the stimuli. The Hog1p is the ultimate MAPK of the Hog1 pathway in *S. cerevisiae* (reviewed by Hohmann, 2007).

The activation of the HOG pathway in response to hyperosmotic stress is initiated by special proteins embedded in the plasma membrane of *S. cerevisiae* that are able to sense changes in osmolarity. Also known as osmosensors, Sho1p and Sln1p control the two parallel branches of the HOG pathway that converge on the MAPKK Pbs2p. The activation of the Sho1 branch is not fully understood, but it has been suggested that changes in the cell shape and/or cell surface conditions may be sensed by Sho1p, which in turn activates the MAPK signaling cascade. Sho1p can interact with downstream signaling elements in the HOG pathway through its C-terminal SH3 domain that can bind and activate Pbs2p (Raitt et al., 2000; Maeda et al., 1995).

In response to osmotic stress, Sho1p forms a transient protein complex at the yeast plasma membrane. Sho1p recruits Pbs2p to the cell surface together with its regulators Ste20p, Ste50p and Cdc42p proteins (Maeda et al., 1995; Posas and Saito

1997; Raitt et al., 2000; Reiser et al., 2000). Ste20p activates the first kinase in line, Ste11p (MAPKKK), and the latter phosphorylates Pbs2p MAPK. Pbs2p phosphorylates the ultimate MAPK Hog1p on both Thr174 and Tyr176 residues. Once in the nucleus, Hog1p is recruited to target promoters by Hot1p, Sko1p, and Msn2p/Msn4p transcription factors (Alepuz et al., 2004; de Nadal et al., 2004; Pokholok et al., 2006).

The Sln1 branch negatively controls the HOG pathway. The *SLN1* gene encodes an enzyme with histidine kinase and aspartate phosphotransferase activities and functions as a plasma membrane sensor. Under non-stressed conditions, Sln1p actively transfers a phosphate to Ypd1p, which in turn phosphorylates Ssk1p (Darin and Gorman, 1999). This phosphorylation inactivates Ssk1p and therefore inhibits the downstream activation of elements in the HOG pathway. Under hyperosmotic stress, Ssk1p is dephosphorylated and therefore can activate the Ssk22p and Ssk2p. These MAPKKK's phosphorylates Pbs2 MAPK to induce the osmoadaptation response through the HOG pathway. The MAPK signaling pathway is also negatively controlled by additional phosphotransferases such as Ptp and Ptc which can deactivate Hog1p (Young et al., 2002).

The expression of *STL1* in response to hyperosmotic stress was shown to be strictly Hog1p-dependent (Westfall et al., 2008). *STL1* encodes for a membrane H^+/glycerol symporter that actively uptakes glycerol into the cell during response to hyperosmotic stress (Ferreria et al., 2005). Alepuz et al. (2001) showed that when *S. cerevisiae* cells were exposed to 0.4 M NaCl, Hog1p kinase was delocalized to the

nucleus to interact with Hot1p transcription factor on the promoter region of the *STL1* gene (Figure 2.2.2.3.1). This association occurs only under hyperosmotic stress conditions (Alepuz et al., 2001). Hot1p is a nuclear protein that seems to control a set of less than 10 genes, including the glycerol synthesis gene, *GPD1* and glycerol transport gene, *STL1*. Deletion of *HOT1* completely abolishes the induction of *STL1* and therefore *STL1* expression directly depends on the activation of the HOG pathway and Hog1p MAPK (Rep et al., 2000; Westfall et al., 2008).

2.2.2.4 Glycerol uptake during hyperosmotic stress

In addition to glycerol synthesis, yeast can also modulate the transport of glycerol across the membrane to increase the level of intracellular glycerol. Evidence shows that hyperosmotic stress results in a decreased rate of glycerol passive diffusion across the plasma membrane, to increase glycerol retention. In addition, yeast can control glycerol transport proteins located in the yeast plasma membrane, Fps1p and Stl1p (Figure 2.2.2.2.5.1) to regulate glycerol efflux and uptake. Fps1p is an aquaporin channel that transports glycerol into or out of the cell via facilitated diffusion. This channel is mainly responsible for the regulation of glycerol efflux and is controlled by gating mechanism and also directly involves the regulation of the N loop which keeps Fps1p in a conformation that allows it to readily restrict glycerol transport in the presence and absence of osmotic stress. In the absence of this domain, transmembrane glycerol flux is too high to allow

efficient accumulation of glycerol by the cell, hence sensitivity to high hyperosmotic stress (Tamás et al., 1999; Tamás et al.,2003).

Under hyperosmotic stress conditions, this channel is shut down within minutes to prevent glycerol leakage (Tamás et al., 1999). In parallel to that, the Stl1p H+/glycerol symporter is induced, to uptake glycerol in an energy dependent manner, by using the proton gradient across the plasma membrane (Ferreria et al., 2005).

2.2.2.5 Controlling the activity of glycerol transport proteins Fps1p and Stl1p

Glycerol can enter *S. cerevisiae* cells using three known mechanisms: passive diffusion, facilitated diffusion via the Fps1p channel and active transport by Stl1p H^+/glycerol symporter (Figure 2.2.2.2.5.1). Each of these mechanisms is used depending on the environmental conditions the cells are exposed to.

Glycerol is an uncharged small molecule with a liposoluble nature that can readily cross the plasma membrane of *S. cerevisiae* via passive diffusion (Blomberg and Adler,1992). Glycerol transport studies with hyperosmotically stressed yeast show a reduction in glycerol simple diffusion rate out of the cell (Sutherland et al., 1997). It appears that yeast limits glycerol permeability across the plasma membrane to facilitate glycerol retention.

In addition to passive diffusion, *S. cerevisiae* uses two membrane transporters to control the glycerol transmembrane flux. These are the Fps1p aquaglyceroporin channel and Stl1p H^+/glycerol symporter. Both of these proteins play an important

role in glycerol accumulation and retention in yeast during response to hyperosmotic stress.

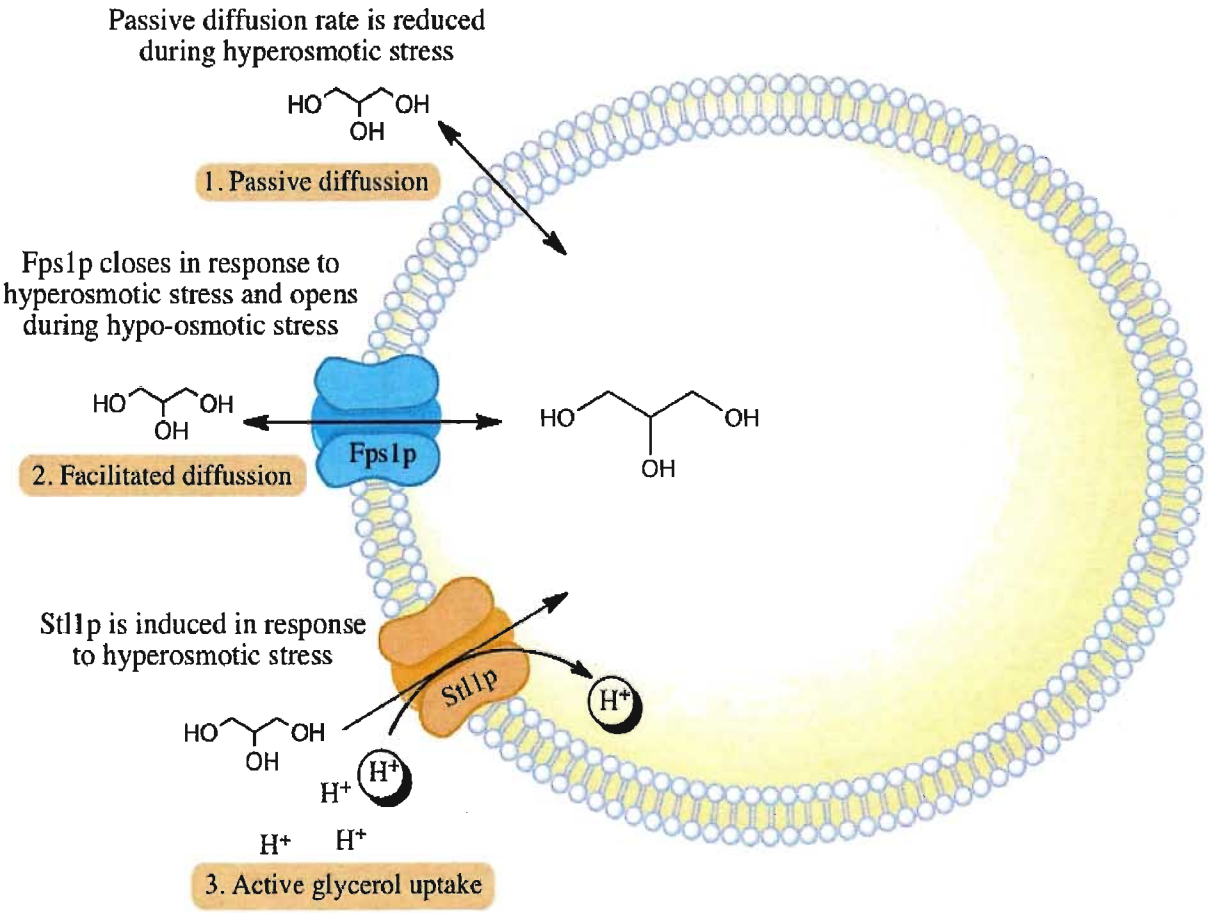

Figure 2.2.2.5.1: Glycerol transport mechanisms in *S. cerevisiae*. Glycerol can enter yeast cell by either (1) passive diffusion, (2) facilitated diffusion mediated by the Fps1p channel and (3) through Stl1p H+/glycerol symporter. (Hohmann, 2009).

Fps1p aquaglyceroporin, encoded by *FPS1* gene, belongs to the Major Intrinsic Protein (MIP) family, which comprise water channels and glycerol facilitators. It is required for glycerol uptake by facilitated diffusion but the major role of Fps1p is controlling glycerol efflux in response to hypo-osmotic shock (Luyten et al., 1995; Tamás et al., 1999). During hypo-osmotic shock, water flows into the yeast cell and causes the cells to swell. Within seconds, cells open the Fps1p channel to allow the release of glycerol. The majority of glycerol accumulated in laboratory strains of *S. cerevisiae* during hyperosmotic shock (80%) is later exported from the cell through Fps1p (Tamás et al., 1999). Conversely, during hyperosmotic stress, the Fps1p channel closes within seconds, to allow glycerol retention (Tamás et al., 2003). The cytosolic N-terminal extension of Fps1p is required for channel closure and its deletion results in unregulated glycerol transport activity (Karlgren et al., 2005). Studies have shown that strains lacking *FPS1* can retain more glycerol than their wild-type counterparts upon salt induced osmotic stress (Luyten et al., 1995; Tamás et al., 2003). Unlike *STL1*, the expression of *FPS1* is not influenced by hyperosmotic stress and therefore is not controlled by the HOG signaling pathway. It is still not clear how *FPS1* transcription is regulated.

STL1 is one of the most upregulated genes in *S. cerevisiae* during response to hyperosmotic stress (Rep et al., 2000; Yale and Bohnert 2001; Posas et al., 2000; Erasmus et al., 2003; Melamed et al., 2008; Martin, 2008). The product of this gene, Stl1p, is an H^+/glycerol symporter that actively uptakes glycerol into the cell during hyperosmotic stress in laboratory strains of *S. cerevisiae* (Ferreria et al., 2005). In

the glycerol-proton symport uptake system, one glycerol molecule is co-transported against its concentration gradient with one proton into the cell. Therefore, glycerol active uptake in yeast will occur only if the proton gradient across the plasma membrane is established.

This symport activity is inhibited in the presence of chemical protonophores such as carbonylcyanide-m-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p* (trifluoromethoxy) phenylhydrazone (FCCP). These are uncharged lipid-soluble weak acids that can cross the yeast membrane, release a proton in the cytosol, and thereby dissipate the proton gradient across the plasma membrane (van Zyl et al., 1990; Lages and Lucas, 1995)

Induction of Stl1p depends on the transcriptional state of *STL1*. Expression of *STL1* is not only induced by hyperosmotic stress in a Hot1p-dependent manner, but also by non-fermentative carbon sources such as glycerol, ethanol and acetate. Yeast can alternate between metabolic pathways in order to utilize these carbon sources when glucose is exhausted in the cell. This phenomenon is also referred to as diauxic shift. Under non-stressed aerobic conditions, glucose represses the expression of *STL1* at the RNA level along with 36 other genes related to ethanol utilization. When glucose is exhausted, cells enter diauxic shift and *STL1* is de-repressed and induced in a Cat8p transcription factor dependent manner (Haurie et al., 2001).

Ferreira et al (2005) were the first to show that Stl1p H^+/glycerol symporter was induced in response to hyperosmotic stress in the aerobically glucose-grown laboratory strain of *S. cerevisiae*. These cells were first grown to the exponential

phase and right after incubated with 0.7 M NaCl. Stl1p induction was detected 30 min after incubation with the salt and peaked after 1.5 hours. Interestingly, Stl1p induction time-line was directly correlated with the level of glycerol uptake rate and accumulation, which also peaked after 1.5 hours of salt stress.

Unlike the rapid induction of Stl1p observed in exponentially grown cells in response to abrupt hyperosmotic stress conditions, cells grown aerobically on glucose and at the same time continually subjected to salt stress (0.7 M NaCl) did not present a rapid induction of Stl1p. Stl1p was detected only after 25.5 hours, at the time point of diauxic shift where glucose was almost exhausted (Ferreria et al., 2005). Therefore, It appears that induction of Stl1p by hyperosmotic stress is detected only when the stress is introduced abruptly after the cells have reached the exponential growth phase (Ferreria et al., 2005).

Interestingly, cells subjected to hyperosmotic stress induced by the high sugar concentration during Icewine fermentation upregulated *STL1* between days 2 to 4 of the fermentation, when glucose levels remain high, yet it is not known what is the role of the product of this gene, Stl1p, in glycerol transport under these anaerobic hyperosmotic stress conditions (Martin, 2008).

2.3 Yeast response to hyperosmotic stress during Icewine fermentation

2.3.1 The effect of Icewine juice on wine yeast K1-V1116 glycerol production and cell growth

Wine yeast fermenting Icewine juice is subjected to hyperosmotic stress due to the high concentration of soluble solids found in the Icewine must. Similar to previous studies with laboratory *S. cerevisiae* strains subjected to salt and sugar stress, Icewine fermenting yeast experiences rapid dehydration, lose membrane turgor pressure and shrink within a few hours upon inoculation. These events eventually lead to slow fermentations and thereby limited cell growth (Kontkanen et al., 2004; Pigeau and Inglis, 2005).

Pigeau and Inglis (2005) studied the response of the wine yeast K1-V1116 to hyperosmotic stress during Vidal Icewine fermentations. To that end, two fermentation conditions were set up, one with Icewine juice containing 40°Brix of soluble solids and the other with diluted Icewine juice containing 20°Brix of soluble solids, comparable to juice used for table wine production. Yeast fermenting Icewine juice experienced greater hyperosmotic stress compared to cells fermenting diluted Icewine juice. This stress resulted in an extended lag phase and a reduced growth rate. Through the course of Icewine fermentation cells doubled only 2-3 times and exhibited a delay in budding. After 48 hours from the time point of inoculation, the cell number in Icewine fermentation media was 3-times lower than the cell number measured in diluted Icewine juice which had already reached mid-exponential growth phase (Pigeau and Inglis, 2005; Martin, 2008). Cells fermenting Icewine juice

accumulated only half of the biomass accumulated by diluted Icewine fermenting cells, even though both cells consumed approximately the same amount of glucose. It appears that during Icewine fermentation, yeast cells are required to re-direct sugar energy towards adaptation to high osmotic stress, rather than utilizing this energy for cell division and growth.

One of the first lines of defense against the dehydration effect of hyperosmotic stress in *S. cerevisiae* is the accumulation and production of glycerol. Yeast cells subjected to hyperosmotic stress can produce glycerol by re-directing a portion of glucose energy from ethanol production to glycerol synthesis. This is also observed during Icewine fermentation. For the same amount of sugar consumed, Icewine cells produce approximately twice as much glycerol compared to cells in the diluted juice fermentations (Pigeau and Inglis, 2005, Pigeau and Inglis, 2007). Interestingly, both Icewine and diluted Icewine fermenting cells release the glycerol to the fermentation media at the onset of fermentation, as observed through monitoring the increase in glycerol concentration in the fermentation media. These results are in contrast to laboratory yeast strain under hyperosmotic stress conditions (Tamás et al., 1999).

2.3.1.1 *Transcriptional response of wine yeast K1-V1116 to hyperosmotic stress and the STL1 gene*

When wine yeast is inoculated into Icewine juice, it remains in lag phase for more than 48 hours. This extended lag phase is required for the yeast to change its metabolism in order to adapt to the hyperosmotic stress through modulating the transcriptional state of osmotolerant related genes. Microarray analysis of the transcriptional response of wine yeast K1-V1116, during Icewine and diluted Icewine fermentation revealed that 2-5% of the genes in the yeast genome were differentially expressed between days 2-5 of the fermentation (Martin, 2008). These include genes that are induced during osmotic stress, among them glycerol synthesis and transport related genes.

Surprisingly, *FPS1*, the gene that encodes for glycerol export protein channel, Fps1p, was not differentially expressed at day 2 of either Icewine or diluted Icewine fermentations and its mRNA transcript could not be detected following that day (Martin, 2008). These results are in contrast with Tamás et al. (1999) who showed that *FPS1* is continually expressed in either saline stressed or non-stressed laboratory yeast cells, thus, its expression is not hyperosmotically stress dependent. It is also interesting to note that when *FPS1* was not expressed, cells were better able to respond to hyperosmotic stress. The same study also found that even though *FPS1* expression is not controlled or induced by hyperosmotic stress, the product of this gene, Fps1p, closes immediately upon exposure to hyperosmotic stress to prevent glycerol leakage from the cell (Tamás et al., 1999).

In the Icewine versus dilute juice fermentation study, *STL1*, the gene that encodes for Stl1p H^+/glycerol symport was found to be the most highly differentially expressed gene between the two fermentation conditions, showing a 25.6 fold difference in expression by day five (Martin, 2008). Furthermore, Northern analysis of *STL1* expression supported microarray results and further revealed that *STL1* was upregulated during the first five days of Icewine fermentation, and its expression peaked on day four for both fermentation conditions (Martin, 2008).

Interestingly, *STL1* appeared in global expression analysis as the most strongly osmostress-induced gene in *S. cerevisiae* when laboratory and wine strains were subjected to different hyperosmotic stress conditions (Rep et al., 2000; Yale and Bohnert, 2001; Posas et al., 2000; Erasmus et al., 2003; Melamed et al., 2008). Sugar-induced osmotic stress upregulated wine yeast *STL1* expression by approximately 87-fold after two hours of exposure to the stress (Erasmus et al., 2003). In laboratory strains of *S. cerevisiae, STL1* was the most or one of the most strongly upregulated genes in response to salt-induced osmotic stress showing 89-fold increase after 10 minutes of treatment with 0.4 M NaCl (Posas et al., 2000). In addition, global analysis of yeast translational response to high salinity revealed that salt stress induced *STL1* mRNA translation (Melamed et al., 2008).

Why do wine yeast fermenting Icewine induce the expression of *STL1*? It is possible that the product of *STL1*, Stl1p H^+/glycerol symporter, has a role in the uptake of glycerol that was present in Icewine juice and continually released to the Icewine fermentation media throughout the fermentation.

2.4 Stl1p H+/glycerol symporter

STL1 is predicted to encode a protein (63.5 kDa) comprised of 569 amino acids with 11 transmembrane domains (SGD). It was first predicted to encode a putative sugar transport-like protein since it displays greatest homology (28% identity) to the products of other sugar transport genes in *S. cerevisiae* such as *HXT2* and *GAL2* (Zhao et al., 1994). The 34 members of this sugar permease protein family include hexose and inositol transporters that transport these metabolites via facilitated diffusion, and members that transport maltose and glycerol using a symport system (Nelissen et al., 1997; Ferreria et al., 2005).

The sugar permease family is part of the Major Facilitator Superfamily (MFS) that include membrane transport proteins with a length of 500-600 amino acids and predicted to comprise 12 transmembrane spanning segments (Nelissen et al., 1997).

Stl1p acts as a H+/glycerol symporter. In this transport system, glycerol uptake completely depends on the proton gradient across the plasma membrane. Glycerol is transported into the cell against its concentration gradient along with one proton molecule. This co-transport system eventually requires the cell to pump protons out of the cell through a membrane ATPase in order to maintain the proton gradient and is therefore considered to be energy consuming. This transport system operates independently of the Fps1p-mediated diffusion (Sutherland et al., 1997).

Active glycerol uptake is differentiated from passive diffusion using a group of chemicals called protonophores. Protonophores, also known as uncouplers, are weak acids that can cross the yeast plasma membrane due to their lipophilic nature

and release a proton in the cytosol (Figure 1.3.1.C). Therefore, chemicals uncouplers such as CCCP can dissipate the proton gradient, and therefore eliminate the energy dependent glycerol entry into the cell.

Lages and Lucas (1997) were first to detect active glycerol uptake in *S. cerevisiae* cells grown on non-fermentative carbon sources such as glycerol, acetate and ethanol. In contrast, fermentative carbon sources such as glucose inhibits active glycerol uptake. Ethanol grown cells shifted to glucose-based media lost their ability to actively uptake glycerol, therefore glucose repressed H^+/glycerol uptake in *S. cerevisiae*, however, only under non-hyperosmotically stressed conditions (Lages and Lucas, 1997). Interestingly, Ferreira et al. (2005) showed that inhibition of active glycerol uptake by glucose is overcome once cells are exposed to hyperosmotic stress. It is not known if cells fermenting Icewine juice induce active glycerol uptake due to the extreme hyperosmotic stress conditions, despite the high concentration of glucose found in the fermentation media.

Kinetic studies of glycerol transport in *S. cerevisiae* revealed that glycerol transport involves two different systems that differ kinetically, one with a high and one with a low Michaelis-Menten values (Km). Glycerol transport measured in cells exposed to concentrations below 10 mM produce low Km values, indicating high affinity to glycerol. This type of saturated kinetics represents the active glycerol transport system. On the other hand, cells suspended in glycerol concentrations higher than 10 mM did not follow saturation kinetics, characteristics of simple diffusion (Lages and Lucas, 1997).

STL1 shows high homology to other proton symporter proteins of the MFS in plant and yeast. These include mannitol symporter in *Apium graveolans* (Noiraud et al., 2001) and sorbitol proton symporter in *Prunus cerasus* (Gao et al., 2003).

Homologues to *STL1* were also found in other yeast species with proton symport activity, these include: *Debaryomces hansenii* (Lucas et al., 1990), the osmotolerant yeast *Zygosaccharomyces rouxii* (van Zyl et al., 1990), *Candida versatilis* (syn. Candida halophila) (Silva-Graçaa and Lucasa, 2006), and the halotolerant yeast *Pichia sorbitophila* (Lages and Lucas, 1997). What is the role of these Stl1p-like proteins in these strains?

Recently, Kayingo et al. (2009) demonstrated that a homologue of *STL1* in *Candida albicans* is required for active glycerol uptake. Unlike glucose-inactivated Stl1p of *S. cerevisiae*, the activity of *C. albicans* glycerol symporter is unaffected by carbon source (Kayingo et al., 2009). Furthermore, *STL1* deletion mutant of *C. albicans* was no more sensitive to salt stress than wild type controls when they were grown in the presence of 1 M NaCl. It is possible that active glycerol transport in *C. albicans* is not the major mechanism used during hyperosmotic stress for glycerol accumulation (Kayingo et al., 2009).

2.4.1 Stl1p glucose inactivation

Under non-hyperosmotically stressed conditions, glucose inhibits active glycerol uptake in *S. cerevisiae* cells. Accordingly, the glycerol symport activity has been described to be under glucose inactivation and can be re-activated when cells are grown on non-fermentable carbon sources (Lages and Lucas, 1997). A study with Stl1p-GFP fusion protein shows that when ethanol-grown cells were shifted to glucose-based medium, plasma membrane Stl1p-GFP was endocytosed and translocated into the vacuole for degradation. The fluorescent signal was no longer detected 1.5 hours after the addition of glucose. Therefore, in the absence of hyperosmotic stress, fermentative carbon source such as glucose activates the degradation of Stl1p, since glycerol is no longer required to be used as a carbon source. This process is achieved by ubiquitination of Stl1p in an *END3* dependent manner (Ferreria et al., 2005). *END3* gene encodes for a protein that is required for the internalization step of proteins during endocytosis. Mutation in the N-terminus of end3p eliminates Stl1p degradation. On the other hand, Stl1p inactivation was not observed in cells grown on glucose and subjected to saline stress. On the contrary, exponentially growing cells induced Stl1p in response to abrupt exposure to 0.7 M NaCl, despite the presence of glucose (Ferreira et al., 2005). It appears that glucose inactivation of Stl1p is overcome when exponentially growing cells are exposed to hyperosmotic stress (Ferreria et al., 2005).

High temperatures also overcome Stl1p-glucose inactivation in *S. cerevisiae*. Ferreira et al (2007) measured active glycerol uptake in yeast grown on glucose at

37°C and 30°C. Even though cells were grown on glucose-based media, active glycerol uptake was still measurable in cells grown at 37°C but not in cells grown on 30°C. Accordingly, Stl1p was not induced at 30°C and was only induced at 37°C.

These findings further emphasize that the regulation of Stl1p –dependent active glycerol uptake in yeast is a dynamic process that depends on the environmental conditions the cells are exposed to. Although cells fermenting Icewine are grown in high glucose and fructose concentration, *STL1* is highly expressed. This expression may lead to Stl1p induction to actively uptake glycerol into the hyperosmotically stressed cells. Therefore we hypothesized that Stl1p glucose inactivation is overcome by the hyperosmotic stress of Icewine fermentation to induce active glycerol uptake.

2.5 *Differences in osmotic stress response are strain, solute and time dependent*

Yeast stress responses are strain specific and may vary under different types of hyperosmotic stress, therefore leading to different metabolic responses (Borneman et al., 2008). These strain differences may influence the response to hyperosmotic stress, specifically in relation to the induction of glycerol transport systems.

The majority of osmotic stress research in yeast has focused on the response of laboratory strains to salt-induced osmotic stress. Laboratory yeast strains exhibit differences in their ability to cope with osmotic stress. Differences are observed in the ability of these strains to ferment sugar. Generally, laboratory strains are unable to effectively ferment wine, in contrast, commercial wine strains are known for their tolerance to ethanol and osmotic stress induced by high sugar concentration (Carrasco et al., 2001). The efficiency to utilize sugar and metabolite production during wine fermentation also varies between commercial strains depending on the level of osmotic stress the cells are exposed to (Carrasco et al., 2001).

Response may also vary depending on the type of solute used to induce the stress. Albertyn et al. (1994) reported that the activity of the glycerol synthesis enzyme Gpd1p in yeast containing the cloned version of *GPD1* was lower in response to salt stress compared to the same level of sugar stress. After three hours of stress, yeast exposed to salt stress produced twice as much glycerol compared to sugar stressed yeast.

Differences in response to hyperosmotic stress are also time-dependent. That is to say, there is variation between short-term and long-term responses. Global transcriptional analysis of a laboratory strain exposed to salt stress show differing expression profiles during the first 10 min, 30 minutes and 90 min of the stress (Yale and Bohnert, 2001). Here, the number of induced genes increased with time from 107 at 10 minutes, to 243 at 30 minutes, then 354 at 90 minutes. Another study shows different results, instead of correlated increase between gene expression and time, the mRNA level of 7% of the upregulated genes in the first 10 minutes of the stress decreased after 20 minutes (Posas et al., 2000). These differences emphasize that gene regulation during early response to hyperosmotic stress is different from that of an adapted response.

The majority of research related to yeast response to hyperosmotic stress has focused on the response of laboratory strains, since their genome has been fully sequenced and knock-out mutants are readily available. Few studies have investigated the transcriptional response of wine yeast during wine fermentation since the genome of a wine yeast strain was not fully sequenced up until 2008 (Borneman et al., 2008), and therefore knockout strains were not available.

Martin (2008) studied the transcriptional response of the wine yeast (K1-V1116) in Icewine and diluted Icewine fermentations. They found that only 28 out of the 186 genes induced in laboratory yeast strain briefly exposed (30-45 minutes) to aerobic salt and sorbitol stress (Rep et al., 2000) were found to be similarly

induced in wine yeast during the first five days of Icewine juice fermentation relative to table wine fermentation.

In 2008, the complete genome of the wine yeast, AWRI1631, was sequenced and compared to the laboratory strain (S288c) (Borneman et al., 2008). It was found that 0.6% of the whole genome and 0.4% of predicted proteome differ between the two studied strains. More specifically, a higher mutation rate was found in sequences coding for proteins that are predicted to be part of the cell wall or signaling transduction pathways (Borneman et al., 2008). Yeast transcriptional response to hyperosmotic stress is mainly regulated through the HOG signaling pathway that upregulates the expression of osmoresponsive genes. Variation in genes related to this pathway may result in different transcriptional response to hyperosmotic stress in wine and lab strains.

These differences may lead to variation between the response of wine yeast and laboratory strains in the glucose repression and inactivation of active glycerol uptake.

2.6 *The interest in Stl1p H+/glycerol symporter in relation to glycerol uptake during Icewine fermentation*

STL1 is highly expressed in wine yeast during Icewine fermentation, however, it is still not known if the product of this gene, the Stl1p H^+/glycerol symporter is active during Icewine fermentation or subjected to glucose inactivation as previously observed in laboratory strains (Ferreria et al., 2005). Because response to hyperosmotic stress may differ depending on yeast strain, solute and duration of the stress, we hypothesize in this project that that wine yeast Stl1p possibly overcomes glucose inactivation during Icewine fermentation. This may be advantageous for yeast adaptation to hyperosmotic stress, since Stl1p may be used to uptake glycerol in order to increase intracellular glycerol levels.

Assuming that Stl1p is not inactivated during Icewine fermentation and actively uptakes glycerol into the cell, can this symport activity lead to the dissipation of the proton gradient across the plasma membrane? Many amino acid transport systems found in yeast are proton symporters, therefore any dissipation of the proton gradient across the cell membrane may reduce amino acid uptake. If this is true, yeast will have to invest energy in order to re-establish the proton gradient and this may lead to limited cell growth. Therefore, the goal of this project is to investigate if Stl1p transports glycerol into the yeast cell during Icewine fermentation in an energy dependent manner. A schematic represenation of the thesis hypothesis is presented in figure 2.6.1.

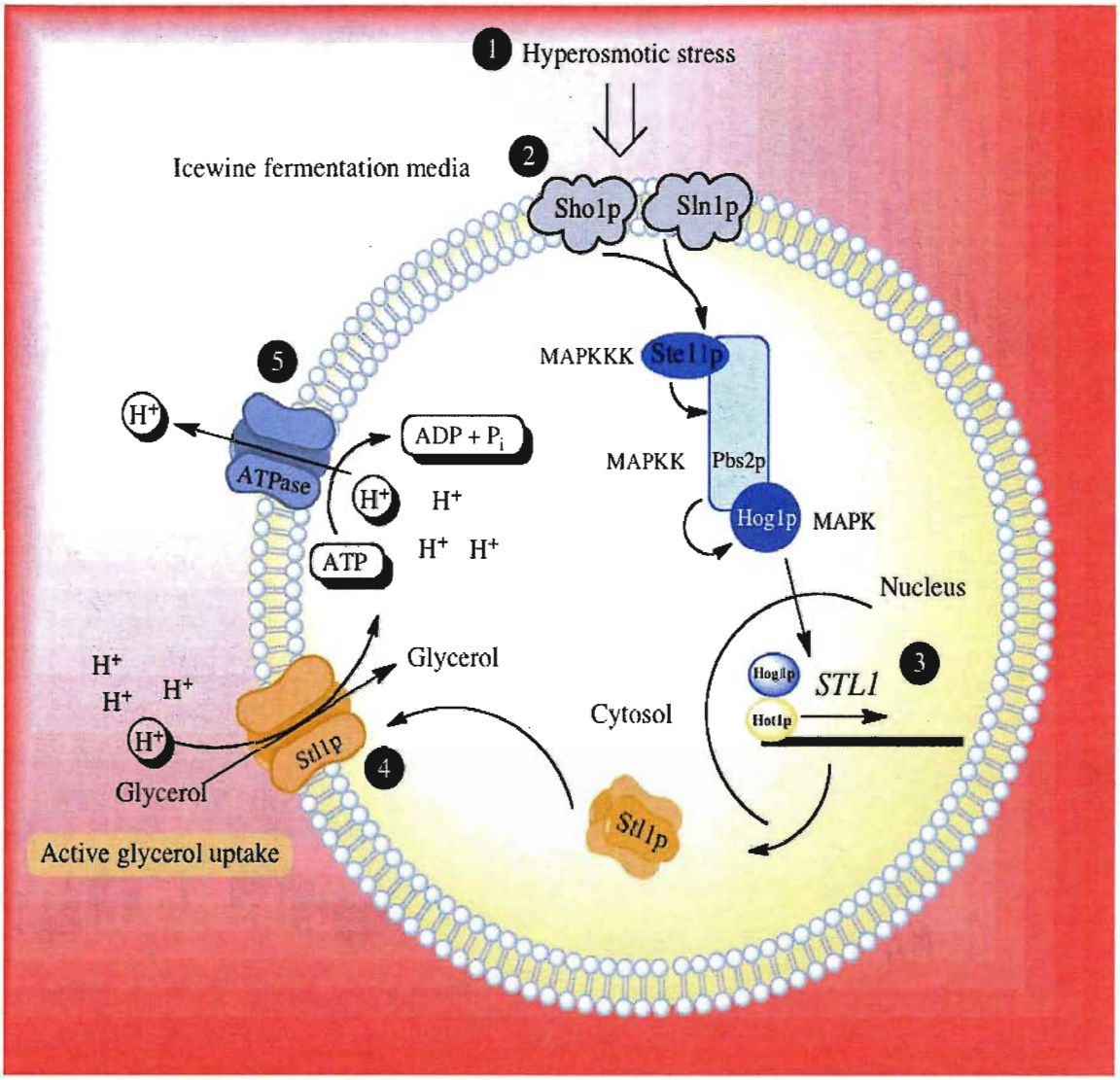

Figure 2.6.1: A schematic representation of thesis hypothesis. (1) Wine yeast inoculated into Icewine juice experiences hyperosmotic stress, which triggers the HOG signaling pathway (2). Activation of the HOG signaling pathway leads to the upregulation of *STL1* and Stl1p induction. (4) Stl1p H^+/glycerol symporter transports glycerol from the fermentation medium into the cell in an energy dependent manner and thereby contributes to the dissipation of the proton gradient across the yeast plasma membrane. (5) Yeast invests energy to restore proton gradient rather than investing it in growth. These events eventually may contribute to the limited cell growth observed during Icewine fermentation. (Hohmann, 2002).

3 MATERIALS AND METHODS

3.1 Yeast Strains

Three yeast strains of *Saccharomyces cerevisiae* were used in this project. The laboratory mutant strain *ΔSTL1* and its parental strain S228C BY4742 (MATα his3D1 leu2D0 lys2D0 ura3D0) obtained from Open Biosystems (Huntsville, AL, USA). The commercial wine yeast strain K1-V1116 was provided by Lallemand Inc. (Montréal, QC, Canada).

3.2 Yeast media for maintaining cultures

S. cerevisiae strains were grown on YPD (2% peptone, 1% yeast extract and 2% dextrose) agar for 3 days at 30°C. The deletion mutant strain, *ΔSTL1* was grown on YPD agar supplemented with 200 μg/mL geneticin (G418) for screening purposes (Sigma; Oakville, ON, Canada). A single colony was inoculated into 3 mL YPD liquid media and grown aerobically for 24 hours at 30°C, 150 rpm to develop the starter cultures for each strain.

3.3 Yeast media and stress conditions for STL1 induction

The starter culture of each strain (300 μL) was inoculated into 100 mL of YEPE medium (2% peptone, 1% yeast extract, 2% ethanol, w/v) supplemented with 0.5% of dextrose. Cells were grown aerobically for 13 hours at 30°C, 150 rpm to an $O.D_{600}$ of 1.5. Hyperosmotic stress was induced by the addition of 25 mL of 5M NaCl to

reach a final concentration of 1M NaCl in the growth media. After 1.5 hours of salt stress, cells were harvested for either Northern analysis or for [^{14}C]glycerol uptake assay.

3.4 Optimizing the conditions for glycerol uptake assay

Throughout the design of glycerol uptake assay different variables had to be adjusted in order to achieve an assay that could produce accurate and reproducible results.

The cell concentration in the reaction mixture was set to 60 mg cells /ml (dry weight) for each reaction. The specific activity (hot glycerol to cold glycerol ratio) for glycerol stock solutions was set to 900, 300 and 53.5 dpm/nmol at 4, 10 and 65 mM glycerol respectively.

The temperature of the reaction was set to 30°C with slow speed stirring to prevent cells from precipitating to the bottom of the reaction tube. In addition, filters were pre-wet to improve water flow during the washing procedure.

A volume of 10 uL of the reaction mixture was removed at specific time points throughout the assay for filtration and the residual extracellular [^{14}C]glycerol was removed by washing the filter with 5 mL of Ice-cold water.

To make certain that the [^{14}C]glycerol was not stuck on the membrane due to its liposoluble nature, the wash step was also tested with a glycerol solution as opposed to water. Results for this optimization procedure appear in appendix 9.2.

3.5 *Preparation of cells for [^{14}C]glycerol uptake assay*

Cells from the salt induced cultures were collected (125 mL) after 90 minutes of stress and pelleted at 3800 g for 5 min at 4°C using Sorvall RC 5C plus centrifuge with SS-34 rotor (Sorvall; Newtown, CT, USA). Pelleted cells were then washed twice with 5 mL of ice-cold 100 mM Tris-citrate buffer (pH 5.0) to remove residual growth media and salt. Cells were harvested in a pre-weighed 2 mL eppendorf tube by centrifugation at 16000 g for 5 min at 4°C using Sorvall RMC-14 centrifuge. The pellet was suspended in 100 mM ice-cold Tris-citrate buffer to a final wet weight concentration of 675 mg of cells/mL (150 mg cells/mL dry weight) and kept on ice until it was used for the [^{14}C]glycerol uptake assay. A volume of 110 ul of this cell suspension was used in the reaction mixture of [^{14}C]glycerol uptake asay to reach a final concentration of 60 mg cells/mL (dry weight).

3.6 *[^{14}C]glycerol stock solutions*

[^{14}C]glycerol uptake was measured using three different glycerol stock solutions: 80 mM (900 dpm/nmol), 200 mM (300 dpm/nmol) and 1.3 M (53.5 dpm/nmol). [^{14}C]glycerol stock solutions were prepared by mixing [^{14}C]glycerol (145 mCi/mmol, GE healthcare, Buckinghamshire, England) and non labeled glycerol (cold glycerol). The volume ratio of labeled and non-labeled glycerol was determined according to the desired specific activity and glycerol concentration of the stock solution.

3.7 [^{14}C]glycerol uptake assays

To measure glycerol uptake in hyperosmotically stressed cells, 80 µL of stock cell suspension was mixed with 110 µL of 100 mM Tris-citrate buffer (pH 5) in a 2 mL eppendorf tube to reach a final concentration of 60 mg cells/mL (dry weight) in the final reaction mixtures. A special temperature controlled chamber was designed (Figure 3.7.1) to hold the tubes above a stirring plate in order to control the speed of stirring. The mixture was incubated at 30°C with gentle magnetic stirring for 2 minutes (Figure 3.7.2). Cells were then incubated with either 1% ethanol (control) or 25 µM of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1.25 mM stock solution, 50% ethanol) to prevent accumulation of glycerol through glycerol/proton symport activity (Figure 1.3.1 C). After 1 minute [^{14}C]glycerol assay was started by the addition of 10 µL [^{14}C]glycerol of either 80 mM (900 dpm/nmol), 200 mM (300 dpm/nmol) or 1.3 M (53.5 dpm/nmol) stock solutions to reach a final glycerol concentration of 4 mM, 10 mM or 65 mM respectively in the final reaction mixture (200 µL final volume). At specific time intervals, 10 µL aliquots were removed and filtered through Whatman GF/C filters (25 mm diameter). The cells were then washed twice while still on the filter with 5 ml ice-cold water to remove residual extracellular glycerol and then the filters were transferred to glass vials containing 5 ml scintillation fluid (Econo Safe, Fisher). [^{14}C]glycerol was counted using a Beckman Coulter scintillation system (LS 6500) and the level of glycerol taken up by the cells was inferred from the amount of [^{14}C]glycerol retained on the filter. Counts

of [^{14}C]glycerol were converted to umoles of glycerol using the next equation:

(Counter efficiency (92%) * Counts per minute) / Specific activity.

Figure 3.7.1: Temperature-regulated chamber that was designed for [^{14}C]glycerol assay in order to hold the reaction tubes.

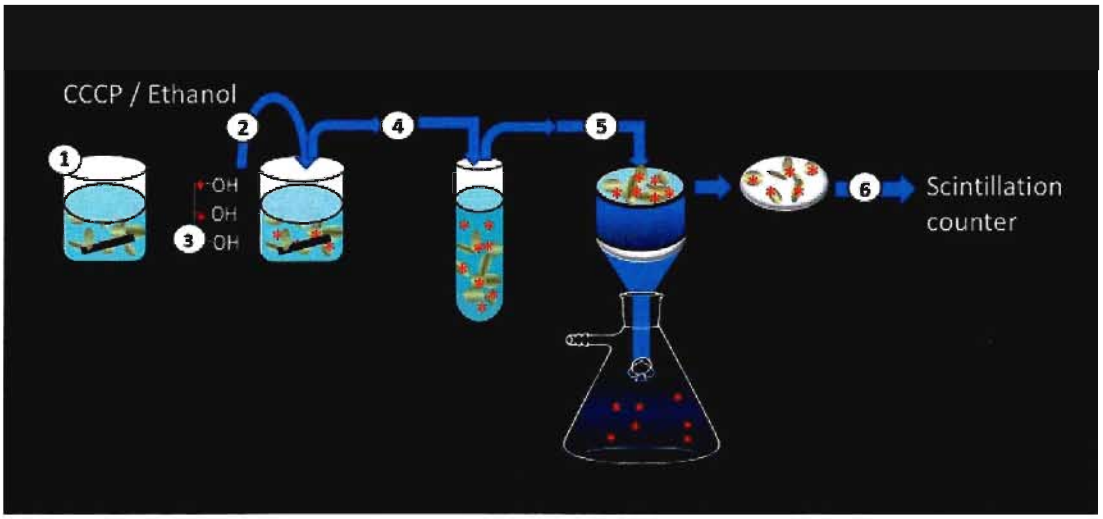

Figure 3.7.2: A schematic representation of [^{14}C]-glycerol uptake assay. (1) Hyperosmotically stressed cells were washed twice and resuspended in 100 mM Tris-citrate buffer to a final concentration of 60 mg cells/ml (dry weight) in reaction mixture and stirred for 2 min at at 30°C. (2) cells were incubated with either 1% ethanol (control) or 25uM CCCP for 1 minute. (3) The reaction was started by the addition of 10 µL of the ^{14}C-glycerol stock solution. (4) At specific time points 10 µL of reaction mixture was aliquoted out and diluted in 5 mL ice-cold water to stop the reaction. (5) Cells were immediately filtered and washed twice with 5 mL ice-cold water to wash off excess [^{14}C]-glycerol. (6) The radioactive glycerol retained on the membrane was counted using scintillation counter. The amount of glycerol up-taken by the cells was inferred from the level of radioactivity retained on the filter.

3.8 Data analysis of [^{14}C]glycerol uptake assays

Glycerol initial uptake rate was determined by calculation of the initial slope of glycerol uptake curve using the first three data points. The level of glycerol accumulation was determined at the time point where the glycerol uptake curve begun to plateau. The % decrease in glycerol accumulation was determined using the following equation:

$$\% \text{ decrease} = \frac{\text{Glycerol}_{-CCCP} - \text{Glycerol}_{+CCCP}}{\text{Glycerol}_{-CCCP}}$$

Each assay was done in triplicate or more and subjected to two-tailed unpaired t-test to determine statistical significant differences in glycerol initial uptake rates and glycerol accumulation (Prism v.3, GraphPad; CA., USA). The results of t-test analysis were presented as either extremely significant (***, $P<0.001$), very significant (**, $0.001<P<0.01$), significant (*, $0.01<P<0.05$) or not significant (NS, $P>0.05$), at the 95% confidence interval.

3.9 *Icewine juice preparation*

Riesling Icewine was kindly provided by Niagara Vintage Harvesters (Virgil, ON. Canada). The juice was filtered through a series of coarse, medium and fine pore-size pads using a Bueno Vino Mini Jet Filter system (Vineco; St. Catharines, ON, Canada). The juice was then sterile-filtered through a 0.22 μm membrane cartridge filter (Millipore; Etobicoke, ON, Canada) into sterile 1L bottles. The sterile juice was stored at -40°C prior to the fermentation experiments.

Table 3.9.1: The initial concentrations of sugars and glycerol in Riesling and Vidal Icewine juice

Parameter	Vidal 38°Brix		Riesling 37°Brix	
	Icewine Juice	Dilute Juice	Icewine Juice	Dilute Juice
Glycerol (g/L)	1.75 ± 0.10	1.102 ± 0.02	2.201 ± 0.05	1.031 ± 0.023
Glucose (g/L)	172.03 ± 4.62	81.70 ± 3.201	198.67 ± 2.65	92.45 ± 0.89
Fructose (g/L)	214 ± 3.3	108.5 ± 4. 702	255.12 ± 4.51	130.05 ± 3.75

3.10 *Perpetration of starter culture for Icewine and dilute Icewine fermentations*

Starter cultures were prepared with the commercial wine yeast *Saccharomyces cerevisiae* K1-V1116 by using a step-wise acclimatization method. In a sterile 250 mL Erlenmeyer flask, 5.0 g of dehydrated yeast was rehydrated with 50 mL of sterile distilled water, at 40°C for 15 min with gentle swirling every 5 min to encourage aeration. Then after, 50 mL of sterile diluted Icewine juice (18.5°Brix Riesling or 19°Brix Vidal) was added aseptically to the rehydrated yeast and the starter culture was incubated at 25°C for 1 hour in a water bath, swirling every 30 min. Next, 50 mL

of undiluted Icewine juice (37 °Brix Riesling or 38°Brix Vidal) was introduced to the starter culture to reach a final concentration of approximately 18.5°Brix (150 mL).

The starter culture was incubated at 20°C for 2 h and the culture was aerated by periodically swirling the flask every 30 min. A sample of the starter culture was examined for actively budding yeast cells under 40x magnification using a light microscope prior to inoculation.

To measure the activity of Stl1p in hyperosmotically stressed Icewine cells, two fermentations were set up: one with undiluted Icewine juice of (37 °Brix Riesling or 38°Brix Vidal), and the control fermentation of diluted Icewine juice (18.5°Brix Riesling or 19°Brix Vidal). The starter culture (7.5 mL) of the starter culture was used to inoculate 0.5 L each of Icewine juice and diluted juice reaching a final yeast inoculation rate of 0.5 g (dry weight) L^{-1}. Fermentations were carried out in sterile fermentation vessels that were fitted with air locks at 17°C. Icewine and diluted Icewine juice fermentations were performed in triplicate.

3.11 Stl1p [^{14}C]glycerol uptake assay of K1-V1116 fermenting Icewine and diluted Icewine juice

Wine yeast cells (K1-V1116) from both fermentation conditions were harvested for Stl1p [^{14}C]glycerol uptake assay at the peak of *STL1* expression in Icewine fermenting cells, as previously determined by northern analysis (Martin, 2008). Fermentation medias were pelleted at 3800 g for 5 min at 4°C using Sorvall RC 5C

plus centrifuge with SS-34 rotor (Sorvall; Newtown, CT, USA). Cells were washed twice with 5 mL of Ice-cold 100 mM tris-cirtrate buffer (pH 5) to remove fermentation media. Cells were then concentrated to 126 mg cells/mL in 100 mM ice-cold Tris-citrate buffer (pH 5.0). 190 µL of cell suspension were incubated at 30°C for two min with stirring and the assay continued as described in section 2.6 at 4 mM glycerol (900 dpm/nmol) in the final reaction mixture. Each assay was performed in triplicate.

3.12 STL1 expression analysis using Northern blots

The expression of *STL1* was analyzed using northern analysis under different stress conditions: (1) in BY4742 and *ΔSTL1* laboratory strains and wine yeast K1V1116 grown on 2% ethanol based media and stressed with 1M NaCl for 1.5 hours, and in (2) K1-V1116 fermenting Icewine and diluted Icewine juice.

Expression of *STL1* in K1-V1116 fermenting Riesling juice was analyzed from days 4 to day 7 and cells fermenting Vidal juice from days 2 to 5.

3.12.1 *STL1* Probe amplification and purification for Northern analysis

The forward and reverse primers of the *STL1* DNA probe were designed by Martin (2008). The forward primer, 5'-TCAAAGGCAAATTTATAAGCAGAAC-3' and reverse primer, 5'-CCAAAATCAATCCAATAAGCAATCA-3' were used to amplify a 550 pb fragment of the *STL1* gene using polymerase chain reaction (PCR). PCR was

carried out in PTC-200 DNAEngine thermocycler (MJ Research; Waltham, MA, USA) using the following parameters: 30 cycles of 94ºC for 40 s, 55ºC for 60 s and 72ºC for 90 s. The PCR product was run on 1% (w/v) agarose gel to verify probe size and was visualized under UV light using BioRad Gel-doc 1000 system. The probe was then purified with a Qiagen QIAquick (Mississauga, ON, Canada) gel extraction kit.

3.12.2 RNA extraction

The RNA of BY4742, *ΔSTL1* and K1-V1116 were extracted using the method of Pigeau and Inglis (2005). RNA was extracted from BY4742, *ΔSTL1* and K1-V1116 grown on ethanol based media (YEP supplemented with 2% ethanol) and from K1-V1116 fermenting Icewine and diluted Icewine juice.

Approximately 50 mL of yeast cells grown on ethanol-based media were removed for RNA extraction. Volumes of 20 to 80 mL were removed from Icewine fermentation media and diluted Icewine fermentation media for RNA extraction of wine yeast K1-V1116.

Removed volumes of both fermentation media and ethanol-based media were supplemented with 0.01 mg mL^{-1} cyclohexamide. Cells were immediately pelleted at 3800 *g* for 5 min at 4°C using a Sorvall RC 5C plus centrifuge (Sorvall; Newtown, CT, USA). The cells were resuspended in 10 mL of cold Diethylpyrocarbonate (DEPC)-treated distilled water and were pelleted as before. While still on ice, cells were resuspended in 375 µL of extraction buffer (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0) and 5% Triton X-100) and 250 µL phenol/chloroform/isoamyl

alcohol (PCI, 25:24:1). Following the addition of 300 mg of glass beads, the suspension was vortexed at 2500 rpm for 6 min to lyse the cells. After vortexing, 6 µL of 20% SDS was added and the mixture was left to stand on ice for 1 h. The mixture was centrifuged at 16000 g in a Sorvall RMC-14 centrifuge for 20 min at 4ºC. To precipitate the RNA, 15 µL of 5 M NaCl and 1250 µL absolute ethanol was added to the supernatant. The solution was allowed to sit at -30ºC for 2 h prior to centrifugation at 16000 g for 20 min at 4°C. The resulting RNA pellet was resuspended in 50 µL of DEPC-treated distilled water and stored at -80ºC until use. RNA concentration was determined spectrophotometrically at 260 nm.

3.12.3 Membrane preparation for northern hybridization

Extracted RNA samples (30 µg) were separated on 1.25 % agarose gel containing 18% (v/v) formaldehyde and blotted onto positively charged nylon membrane (Roche; Basel, Switzerland) using capillary transfer. The RNA was cross-linked to the membrane using Hoefer UVC 500 crosslinker for 4 min, for each side of the membrane (Hoefer, Inc.; CA, USA).

3.12.4 *STL1* probe labeling and hybridization

The *STL1* probe was labeled with [α-^{32}P]-ATP (perkin Elmer) using a Roche random primed labeling kit (Roche; Basel, Switzerland) and 20 ng of the labeled probe was hybridized to the bound RNA following an overnight incubation at 42°C

in 50 mL of hybridization solution containing 5X SSC, 5X Denhardt's solution, 1% SDS, 50% (v/v) formamide and 0.1 mg mL^{-1} sheared salmon sperm DNA (Ambion). The membrane was washed twice with 50 mL 2X SSC containing 0.1% (v/v) SDS for 20 min at room temperature and then washed twice with 50 mL 0.2X SSC, also containing 0.1% (v/v) SDS, for 15 min at 50°C. The probe membrane was exposed to a Fujifilm phosphorimaging screen for up to seven days and the screen was scanned with Fujifilm FLA-3000 phosphorimager (663 nm helium-neon laser) and personal molecular imager™ (PMI™, 635 nm). The subsequent images were quantified with Fujifilm Image Gauge software (v.4.0) and Quantity One software (v.4.6.7). Gene expression levels were normalized to the signal intensities derived from a rDNA region spanning the 5.8S rRNA gene and flanking internal transcribed spacers (ITS) 1 and 2.

3.13 Sequencing the STL1 gene in S. cerevisiae strains

The STL1 encoding region of BY4742 and K1-V1116 was amplified by PCR using forward 5'-ATGAAGGATTTAAAATTATCGAATT-3' and reverse primer 5'-TCAACCCTCAAAATTTGCTTTATCG-3' and carried out as described in section 2.10.1. The PCR product was run on 1% (w/v) agarose gel to verify gene size and was visualized under UV light using BioRad Gel-doc 1000 system. Amplification products were then purified with a Qiagen QIAquick PCR purification kit.

The purified *STL1* PCR (approximately 1700 pb) product was run on 1% agarose gel and quantified using HighRanger 1 kb DNA Ladder (Norgen Biotek, St.

Catharines. ON., Canada) and sent to Génome Québec Innovation Centre (McGill University) for DNA sequencing. Primers for *STL1* were designed by the author and obtained from Sigma Genosys (Oakville, ON., Canada). Two of the designed primers flank the *STL1* (Forward [1-25] and Reverse [1685-1710]) and the other two primers are located at the center region of the gene (Forward [905-930] and Reverse [526-551]). Primer sequences are listed in table 3.12.1. DNA sequences were manually edited and were submitted to ClustalW2 (EMBL-EBI) for alignment. The genes were annotated using CLC Main Workbench v.5.

Table 3.12.1: The Primer sequences used for *STL1* sequencing designed using the laboratory strain of *S. cerevisiae*

Primer	Sequence 5' to 3'
Forward [1-25]	***1***-ATGAAGGATTTAAAATTATCGAATT-***25***
Forward [905-930]	***905***-AGCAATTTACTGGTTGTAACGCTGC-***930***
Reverse [1685-1710]	***1685***-TCAACCCTCAAAATTTGCTTTATCG-***1710***
Reverse [526-551]	***526***-CCAAAATCAATCCAATAAGCAATCA-***551***

4 RESULTS

Part 1-Development of Stl1p-dependent glycerol uptake assay

To develop a glycerol uptake assay that is specifically dependent on Stl1p activity it was first necessary to determine the induction conditions under which *STL1* gene is expressed in parent and wine strain of *S. cerevisiae*. To do that, the expression of *STL1* was investigated under saline stress using Northern blot analysis (section 4.1). Once *STL1* gene expression was confirmed, the same stress conditions were used to induce Stl1p and measure the level of [^{14}C]glycerol uptake in wine and laboratory yeast strains (Section 4.2). Glycerol uptake was measured in saline stressed cells incubated with either 4 mM, 10 mM or 65 mM glycerol in the reaction mixture. The first two concentrations were adapted from a previously published study (Ferreria, et al., 2005) and the latter is the glycerol concentration that was found in Icewine fermentation media at the time point when *STL1* expression peaked. After completing the design of the assay, the assay was applied to measure glycerol uptake in hyperosmotically stressed wine yeast cells, harvested from Icewine fermentation.

4.1 Stl1p induction in wine and laboratory yeast strains

To induce Stl1p in laboratory and wine yeast strain, cells were grown on 2% ethanol (Section 3.2) since it was previously demonstrated that active glycerol uptake is highly induced in *S. cerevisiae* by non-fermentative carbon sources such as ethanol (Lages & Lucas, 1997).

Exponentially growing cells were subjected to 1 M salt stress for 1.5 hours (Section 3.2) and the level of *STL1* expression was compared between hyperosmotically stressed and non-stressed cells using Northern blot analysis (Section 3.11).

Northern blots of *STL1* revealed that the gene was slightly expressed in wine and laboratory yeast strains under non-stressed conditions when grown on 2% ethanol (Figure 4.1.1.A). Upon incubation with 1M NaCl, *STL1* was induced in both parent and wine strains however at different expression levels.

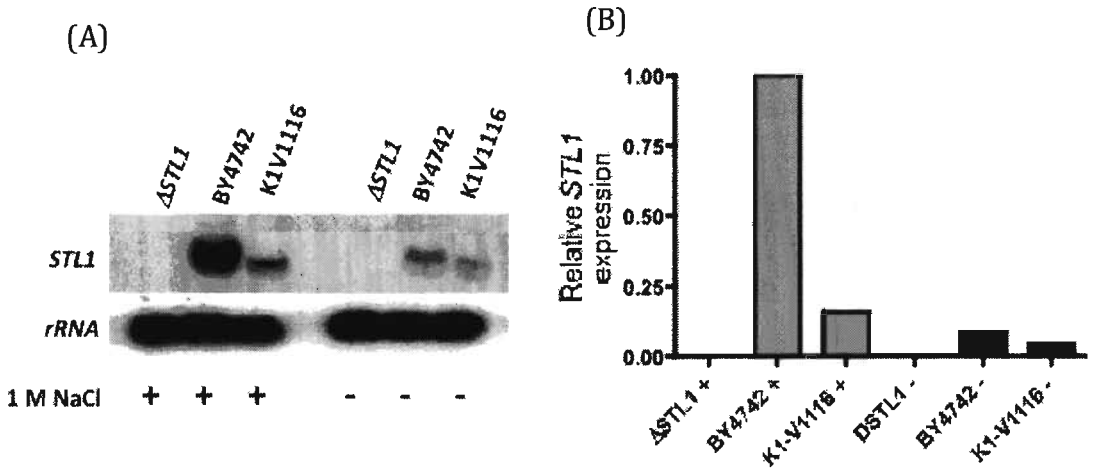

Figure 4.1.1: (A) Northern analysis of *STL1* expression in ethanol grown wine (K1V1116) and laboratory yeast strains (BY4742 and *ΔSTL1*) before and after 1.5 hours of hyperosmotic stress using 1 M NaCl. (B) Relative expression of *STL1*.

The laboratory strain showed an approximately 11.7-fold increase in *STL1* expression compared to a 3.4 fold increase in the wine strain (Figure 4.4.1.B). As expected, *STL1* was not expressed in *ΔSTL1* knockout under either stressed or non-stressed conditions.

These results first confirm that the Northern blot expression signal is specific for *STL1* since a signal is only present in the parent strain and lacking in the *ΔSTL1.* Secondly, the chosen hyperosmotic stress conditions are adequate to induce *STL1* and therefore could be used for [^{14}C]glycerol uptake assay.

4.2 [^{14}C]glycerol uptake at 4 mM glycerol

Glycerol uptake by parent and *ΔSTL1* strains was first measured at 4 mM glycerol concentration in the reaction mixture using the assay outlined in section 3.7, over a 10 minutes time course (Figure 4.2.1).

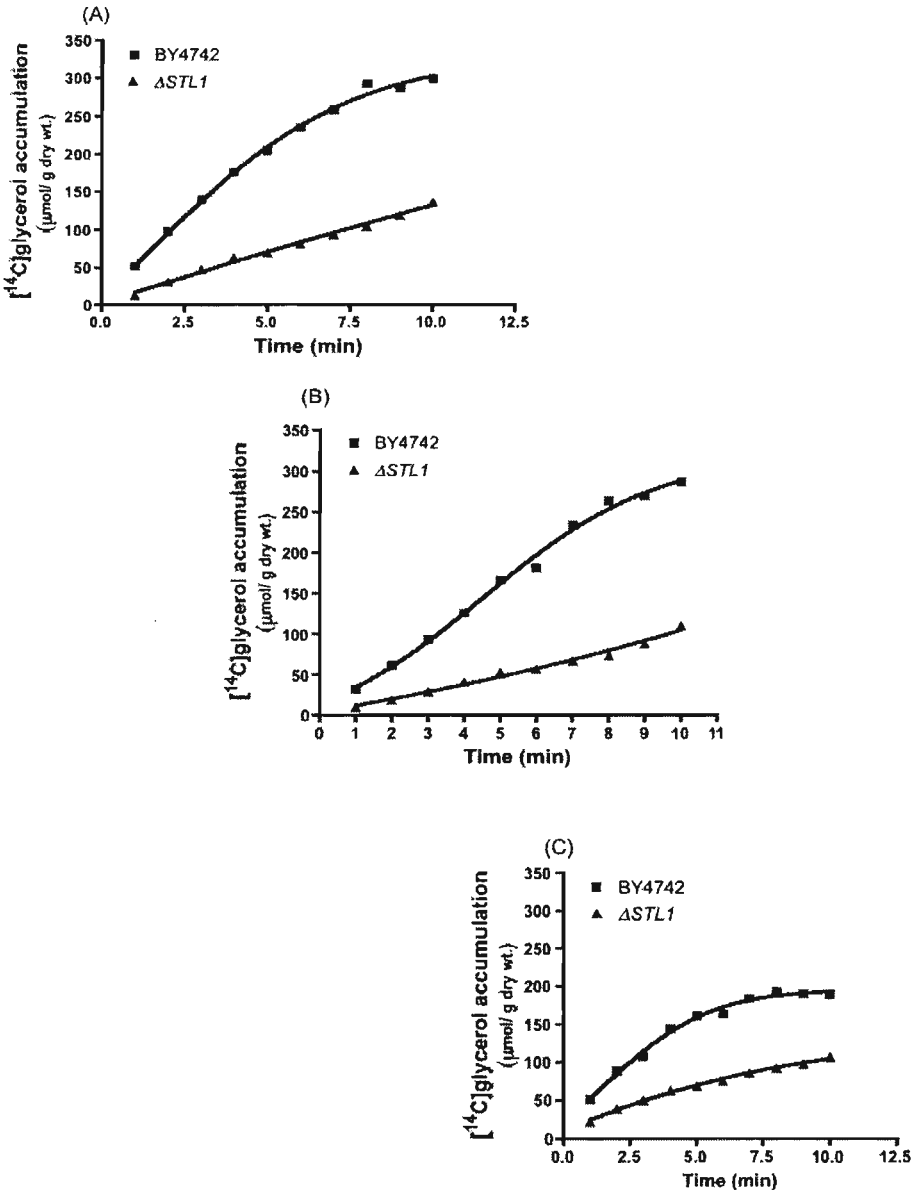

Figure 4.2.1: [^{14}C]glycerol uptake at 4 mM glycerol by BY4742 and *ΔSTL1* from three different cell preparations (A, B and C). Cells were grown on 2% ethanol and stressed for 1.5 hours with 1 M NaCl.

All three separate experiments under 4 mM glycerol conditions presented similar glycerol uptake trends. The parent strain accumulated higher levels of glycerol compared to the *ΔSTL1* knockout. In addition, the kinetics of glycerol uptake by the parent strain was different than that of the knockout strain (Figure 4.2.1). The initial glycerol uptake rate in the parent strain plateaued after approximately 7 minutes. Therefore, glycerol accumulation in parent strains follows a biphasic kinetics.

On the contrary, *ΔSTL1* did not display changes in glycerol transport rate over the 10 minutes of the assay and presented rather a constant glycerol uptake rate, hence, the knockout strain displayed a monophasic kinetics of glycerol uptake.

Comparison between the initial uptake rates of glycerol transport between the two strains revealed that the parent strain was able to transport glycerol into the cell at an initial rate that was 2.6-fold greater than that of *ΔSTL1* (Figure 4.2.2.B).

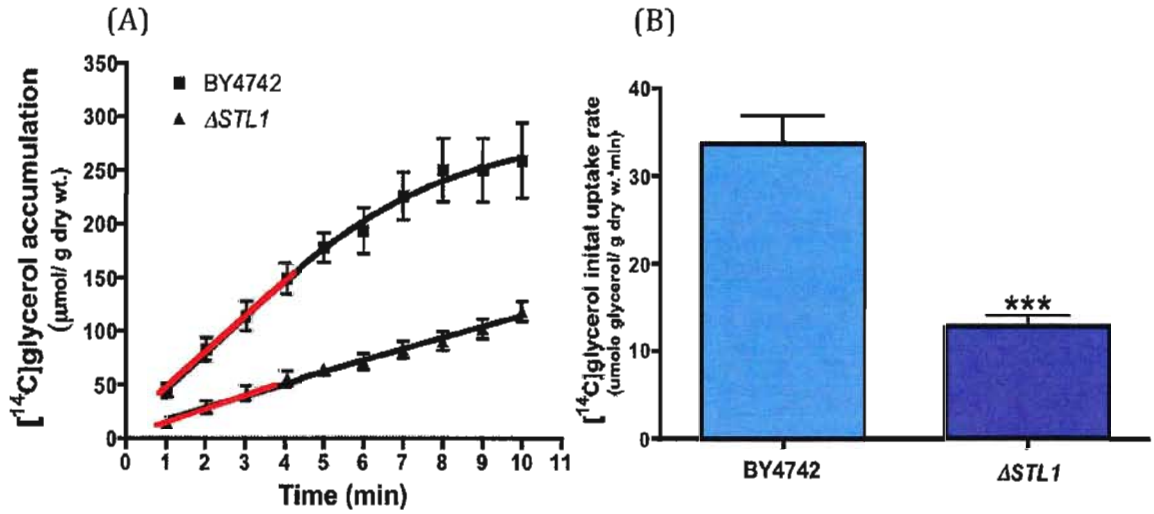

Figure 4.2.2: [^{14}C]glycerol uptake and initial glycerol uptake rate at 4 mM glycerol in BY4742 and *ΔSTL1*. (A) Average of [^{14}C]glycerol uptake (n=3). (B) Comparison of initial glycerol uptake rate. *** The difference between the means was extremely significant at the 95% confidence interval determined by unpaired t-test (n=5, P=0.0004).

These results suggest that the absence of *STL1* reduces the rate of glycerol uptake and therefore the expression of this gene positively contributed to rapid glycerol uptake in hyperosmotically stressed cells.

To examine if glycerol uptake depends on the proton gradient across the yeast plasma membrane, the assay was repeated in the presence of the protonophore CCCP.

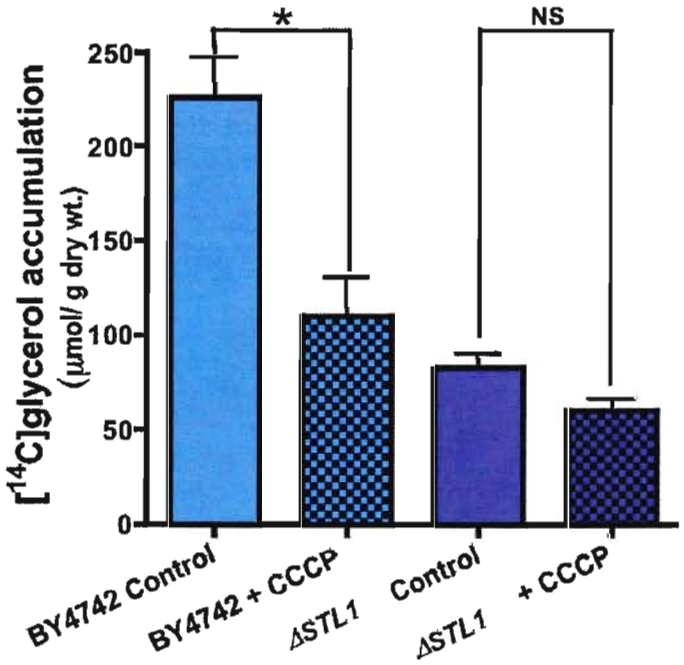

Figure 4.2.3: [^{14}C]glycerol accumulation in BY4742 and *ΔSTL1* at 4 mM glycerol after 7 minutes with and without the incubation with CCCP. * The difference between the means was significant at the 95% confidence interval as determined by unpaired t-test (n=3, P=0.018). NS- means are not significantly different as determined by unpaired t-test.

Incubation with CCCP significantly lowered glycerol accumulation in the parent strain compared to control conditions where no CCCP was added (Figure 4.2.3), indicating the glycerol accumulation in the presence of CCCP was not significantly different to than that measured in the *STL1* strain. Therefore, CCCP inhibited energy-dependent glycerol uptake through Stl1p. In contrast, the level of glycerol accumulation in *ΔSTL1* cells did not significantly change in the presence of CCCP compared to control conditions and therefore did not show energy dependency.

The decrease in glycerol accumulation in the parent strain with CCCP constitutes about 50% of the glycerol that was accumulated under control conditions for the parent strain and therefore represents the contribution of Stl1p in glycerol accumulation in hyperosmotically stressed cells (Figure 4.2.4).

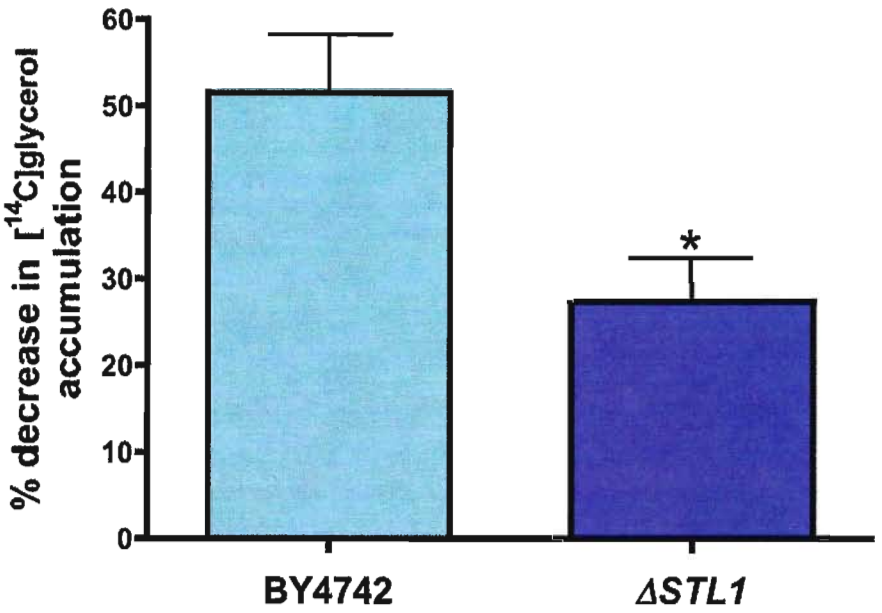

Figure 4.2.4: The % decrease from control in [^{14}C]glycerol accumulation at 4 mM glycerol following the incubation with CCCP after 7 minutes. * % decrease in glycerol accumulation was significantly different than parent strain at 95% confidence interval as determined by unpaired t-test (n=3, P=0.0459).

Since the parent strain exhibited a significantly greater reduction in glycerol accumulation in the presence of CCCP compared to *ΔSTL1*, it is inferred that Stl1p positively contributes to energy dependent glycerol uptake in hyperosmotically stressed cells at 4 mM glycerol.

4.3 [^{14}C]glycerol uptake 10 mM glycerol

The second [^{14}C]glycerol uptake assay was carried out at 10 mM glycerol (Ferreria, et al., 2005) in the reaction mixture, for 60 minutes (section 3.7).

At 10 mM glycerol, the parent strain and the *ΔSTL1* knockout presented glycerol uptake trends that were similar to the 4 mM glycerol assay results. The parent strain exhibited fast glycerol uptake for the first 10 minutes of the assay that begun to decrease after approximately 15 minutes. On the other hand, glycerol uptake by *ΔSTL1* knockout maintained the same rate throughout the course of the assay and therefore represents the passive diffusion rate (Figure 4.3.1.A).

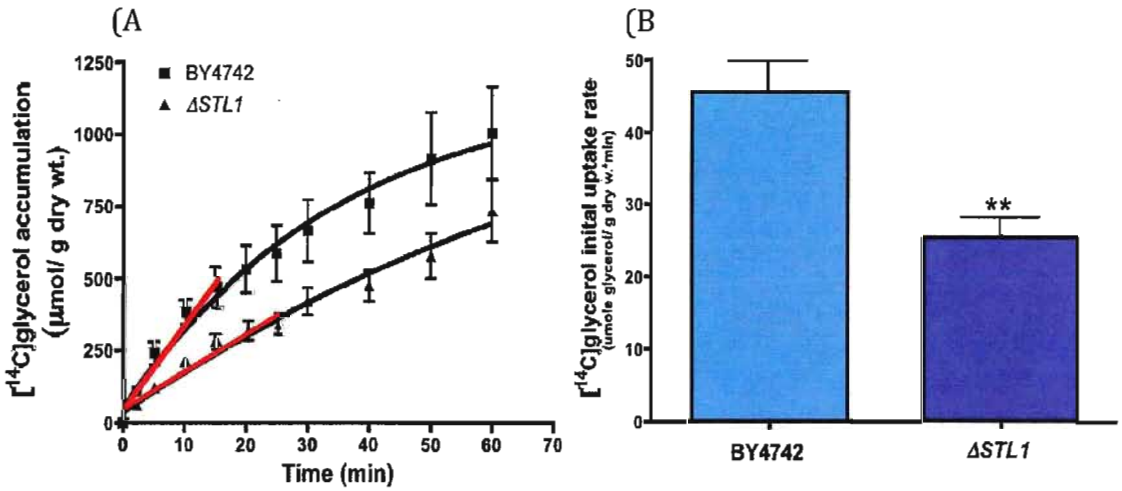

Figure 4.3.1: [^{14}C]glycerol uptake and initial glycerol uptake rate at 10 mM glycerol in BY4742 and *ΔSTL1*. (A) Average [^{14}C]glycerol uptake (n=3). **(B)** Comparison of initial glycerol uptake rate. ** The difference between the means was very significant at the 95% confidence interval as determined by unpaired t-test (n=5, P=0.0066).

Incubation with CCCP significantly reduced the level of glycerol accumulation after 10 minutes in the parent strain (Figure 4.3.2) thereby inhibiting active glycerol uptake, however CCCP also significantly reduced glycerol accumulation in the knockout strain, suggesting that active glycerol uptake was inhibited in a strain lacking the Stl1p symporter. These results may imply the existence of other potential glycerol symporters in *S. cerevisiae* that have yet to be discovered in the known genome.

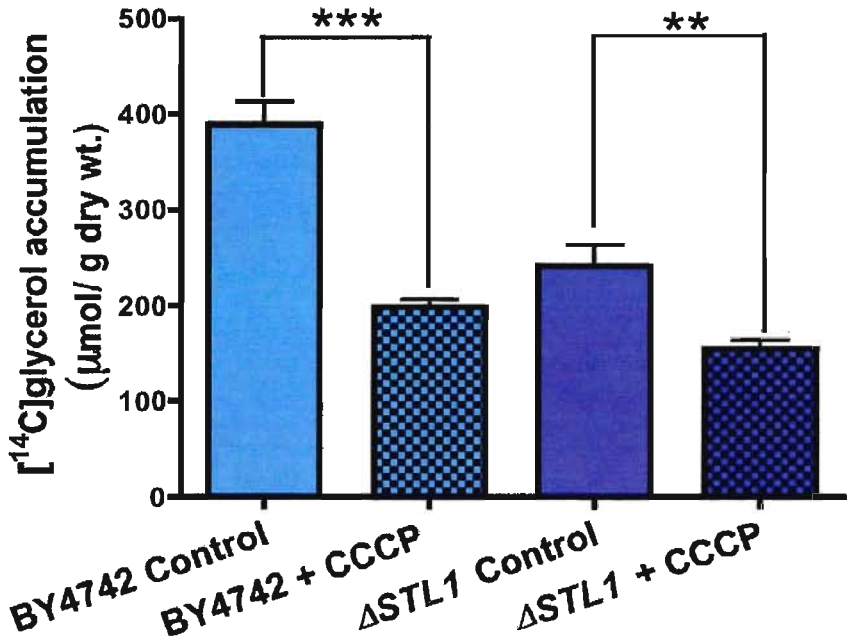

Figure 4.3.2: [^{14}C]glycerol accumulation in BY4742 and *ΔSTL1* cells at 10 mM glycerol after 10 minutes with and without the incubation with CCCP. *** The difference between the means was extremely significant at the 95% confidence interval as determined by unpaired t-test (n=5, P<0.0001). ** The difference between the means was very significant at the 95% confidence interval as determined by unpaired t-test (n=5, P=0.0065).

It is induced from these results that glycerol accumulated in *ΔSTL1* represents background levels of glycerol, that is to say, glycerol that entered the cell through passive diffusion or other unidentified glycerol transport proteins and therefore could not be attributed to Stl1p activity.

Both parent and *ΔSTL1* displayed the same % of glycerol reduction upon incubation with CCCP, hence, sharing similar sensitivity to the protonophore at 10 mM glycerol (Figure 4.3.3). It appears that at concentrations higher than 4 mM glycerol, uptake becomes less Stl1p specific due to increased background levels of glycerol.

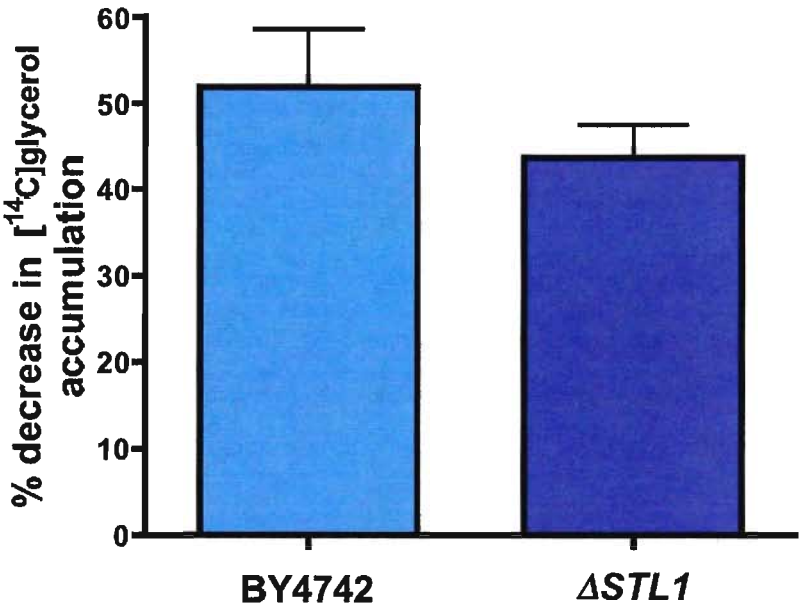

Figure 4.3.3: The % decrease from control in [^{14}C]glycerol accumulation at 10 mM glycerol following the incubation with CCCP after 10 minutes.

To further investigate the degree of Stl1p contribution to glycerol accumulation during the yeast response to hyperosmotic stress, glycerol accumulation was compared between stressed and non-stressed parent strain cells (Figure 4.3.4).

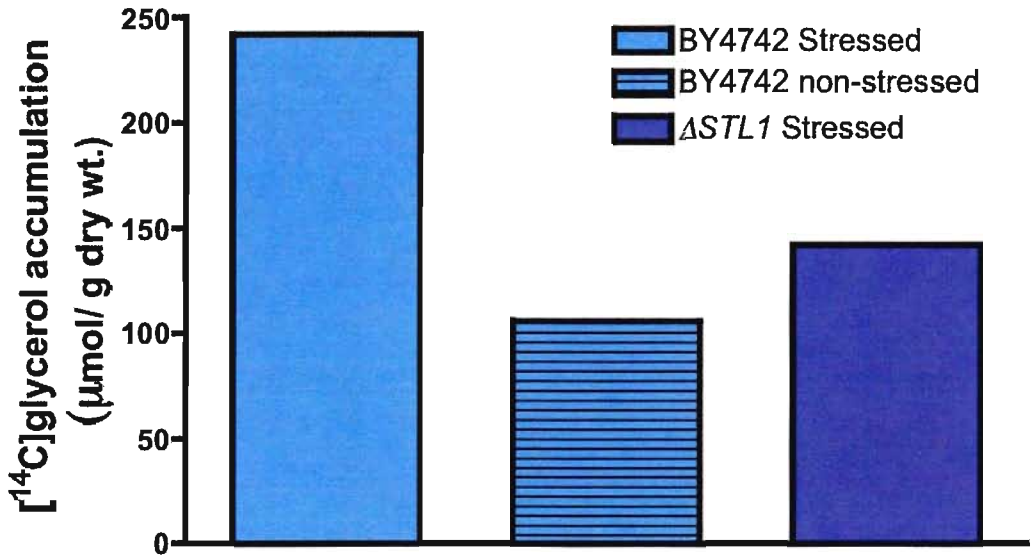

Figure 4.3.4: Induction of [^{14}C]glycerol uptake in BY4742 following saline stress at 10 mM glycerol. BY4742 were either stressed by 1 M NaCl for 1.5 hours or not subjected to saline stress (n=1).

Parent strain cells showed an increase in glycerol accumulation upon exposure to saline stress. This increase was directly correlated to the increase in *STL1* expression as shown in figure 4.1.1. Saline-stressed parent yeast cells accumulated about 2.3 times more than non-stressed parent cells and twice as much glycerol compared to the knockout strain. Once again, it appears that the background levels of non Stl1p-specific glycerol entry into the cell constitute approximately half of the glycerol measured in the hyperosmotically stressed parent cells under the 10 mM glycerol conditions, similar to the results observed under the 4 mM glycerol assay.

These results put further emphasis on the degree of Stl1p contribution for glycerol accumulation in hyperosmotically stressed cells. Stl1p induction by saline stress increases glycerol accumulation by approximately 2-fold.

4.4 [^{14}C]glycerol uptake at 65 mM glycerol

To be able to measure the activity of Stl1p in cells suspended in Icewine fermentation media, it was first necessary to test if the designed [^{14}C]glycerol uptake assay still shows specificity for Stl1p at higher glycerol concentrations as observed in the starting glycerol concentration in Icewine juice and throughout the fermentation. Therefore, the competency of glycerol uptake assay to measure Stl1p-dependent uptake at 65 mM was investigated under the same growth and stress conditions as 4 and 10 mM glycerol assays (section 3.7).

At 65 mM, glycerol uptake by parent strain cells grown on ethanol-based media and exposed to saline stress was fairly constant throughout the course of the assay and actually resembled *ΔSTL1* glycerol uptake trend (Figure 4.4.1.A). The difference in the initial glycerol uptake rate that was previously observed under 4 and 10 mM glycerol concentrations was no longer detected at 65 mM. It is possible that when the reaction mixture contains high glycerol concentration, the contribution of Stl1p to the total intracellular glycerol accumulation is masked by high background uptake, probably due to passive diffusion and/or other uncharacterized glycerol transport systems.

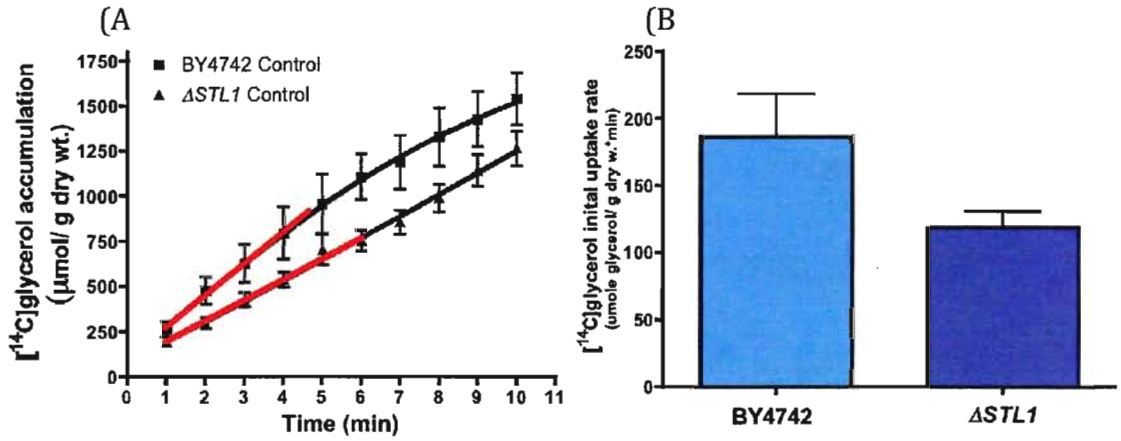

Figure 4.4.1: [^{14}C]glycerol uptake and initial glycerol uptake rate at 65 mM glycerol in BY4742 and *ΔSTL1*. (A) Average [^{14}C]glycerol uptake (n=6). **(B)** Comparison of initial glycerol

In contrast to the 4 mM and 10 mM glycerol assays, glycerol initial uptake rate was not significantly different between parent and *ΔSTL1* under the 65 mM conditions (Figure 4.4.2). Furthermore, the addition of CCCP only significantly reduced glycerol accumulation in *ΔSTL1* and did not significantly affect the parent strain. Even though Stl1p may actively transport glycerol into the cell under 65 mM glycerol conditions, this transport cannot be accurately measured due to the high levels of non Stl1p-specific uptake of glycerol.

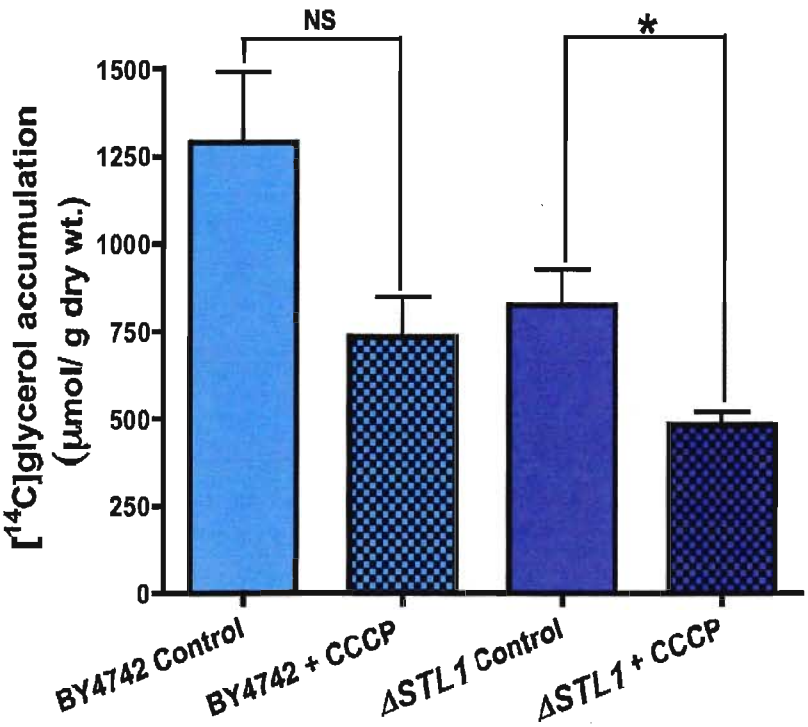

Figure 4.4.2: [^{14}C]glycerol accumulation in BY4742 and *ΔSTL1* at 65 mM glycerol after 7 minutes with and without the incubation with CCCP. * Glycerol accumulation was significantly different than control at 95% confidence interval as determined by unpaired t-test (n=4, P=0.028). NS- means are not significantly different as determined by unpaired t-test (P= 0.053).

All six repeats of 65 mM assay were done from six different cell preparations from six different growth experiments. These six repeats showed variability in glycerol uptake rate and in the level of glycerol accumulation. It was necessary to determine why the difference in initial uptake rate between parent strain and knockout was observed only at lower glycerol concentrations (4 mM and 10 mM) but not at 65 mM glycerol. Did it arise from the high glycerol concentration found in the reaction mixture or due to the high variability among the different growth batches? To investigate this question [^{14}C]glycerol uptake was compared between 4

mM and 65 mM assays in hyperosmotically stressed cells that were harvested from the same growth batch. Results show that glycerol uptake curve of parent strain was almost identical to *ΔSTL1* at 65 mM glycerol (Figure 4.4.4).

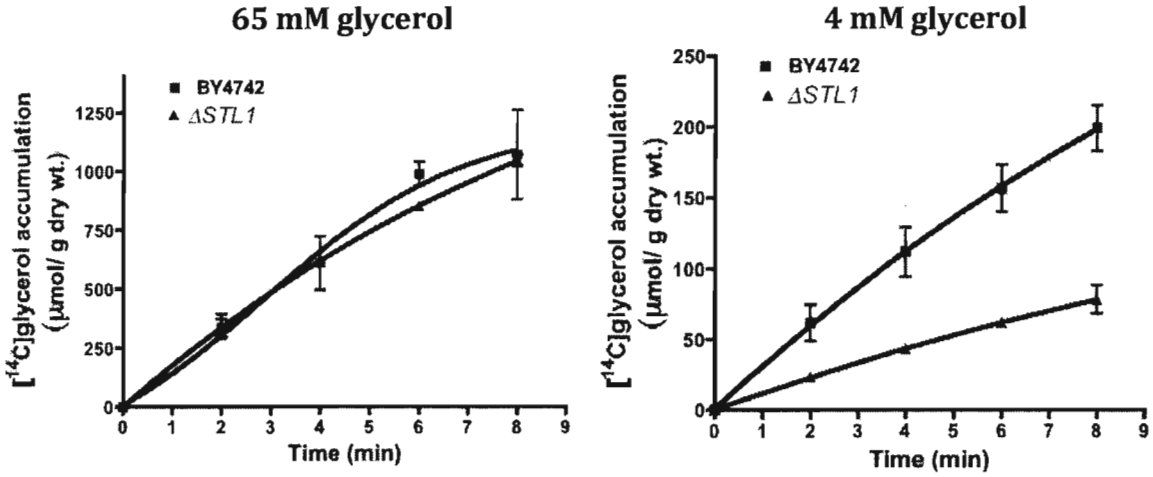

Figure 4.4.4: Comparison of [^{14}C]glycerol uptake of BY4742 and *ΔSTL1* under (A) 65 mM and (B) 4 mM in cells harvested from the same growth batch (n=2).

On the other hand, under 4 mM glycerol, parent strain harvested from the same growth batch presented a faster uptake rate of glycerol compared to *ΔSTL1* as previously presented in section 5.2.

Despite the fact that both 4 and 65 mM assays were performed with cells collected from the same growth batch, the glycerol uptake rate and accumulation by parent strain was only higher and significantly different from the *ΔSTL1 strain* at the 4 mM glycerol assay condition.

It is implied from these results that the glycerol concentration in the reaction mixture greatly influences the competence of the assay to measure Stl1p-dependent glycerol uptake, rather than the variability among the different cell growth batches.

The reason why Stl1p-dependent glycerol uptake was not detected at 65 mM could be related to the high background levels of glycerol that perhaps eliminated the difference in glycerol uptake rate and accumulation. Hence, at 65 mM Stl1p-dependent glycerol uptake could not be accurately measured under these assay conditions.

Comparison between the initial uptake rates measured at 4, 10 and 65 mM revealed that glycerol uptake rate increased with glycerol concentration for both parent and knockout strain (Figure 4.4.5). However the initial uptake rate was no longer significantly different between the strains at 65 mM. This demonstrates that under higher glycerol concentrations, the assay cannot display the contribution of Stl1p to the measured glycerol uptake, since it cannot be distinguished from the background glycerol uptake in the *ΔSTL1* strain.

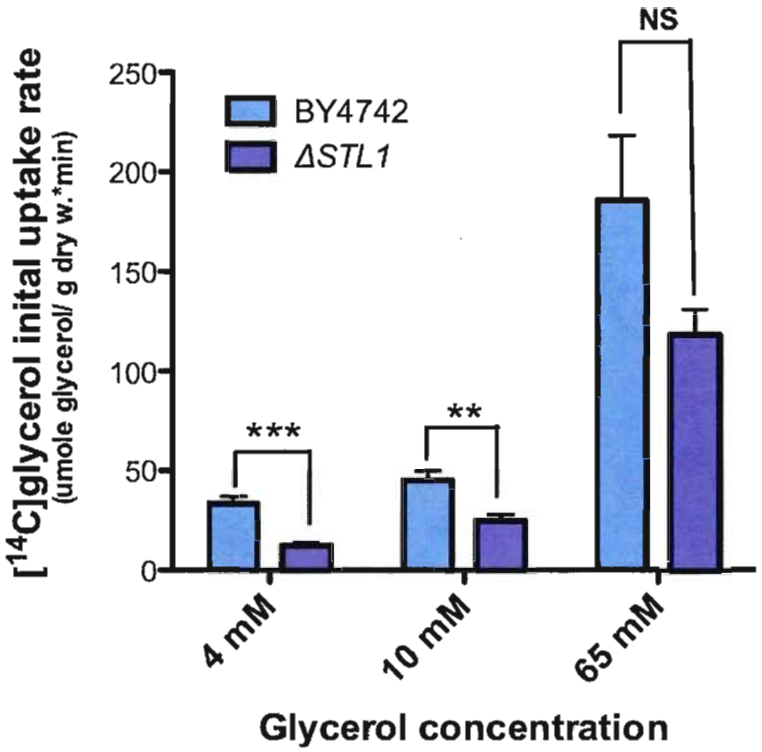

Figure 4.4.5: The effect of glycerol concentration on glycerol initial uptake rate in BY4742 and *ΔSTL1* cells. *** The difference between the means was extremely significant at the 95% confidence interval (n=5, P=0.0004). ** The difference between the means was very significant at the 95% confidence interval (n=5, P=0.0066). NS- No significant difference between the means.

4.5 [^{14}C]glycerol uptake by wine yeast strain at 4 and 65 mM glycerol

Under 4 mM assay conditions the incubation of salt stressed wine yeast with CCCP resulted in a significant reduction in glycerol accumulation as previously observed for parent strain (Figure 4.5.1). On the contrary, at 65 mM glycerol, the addition of CCCP did not significantly effect glycerol accumulation in wine yeast as observed for parent strain. Similar to parent strain, at high glycerol concentration,

Stl1p-dependent active glycerol transport could not be measured in wine yeast using the conditions of the designed assay.

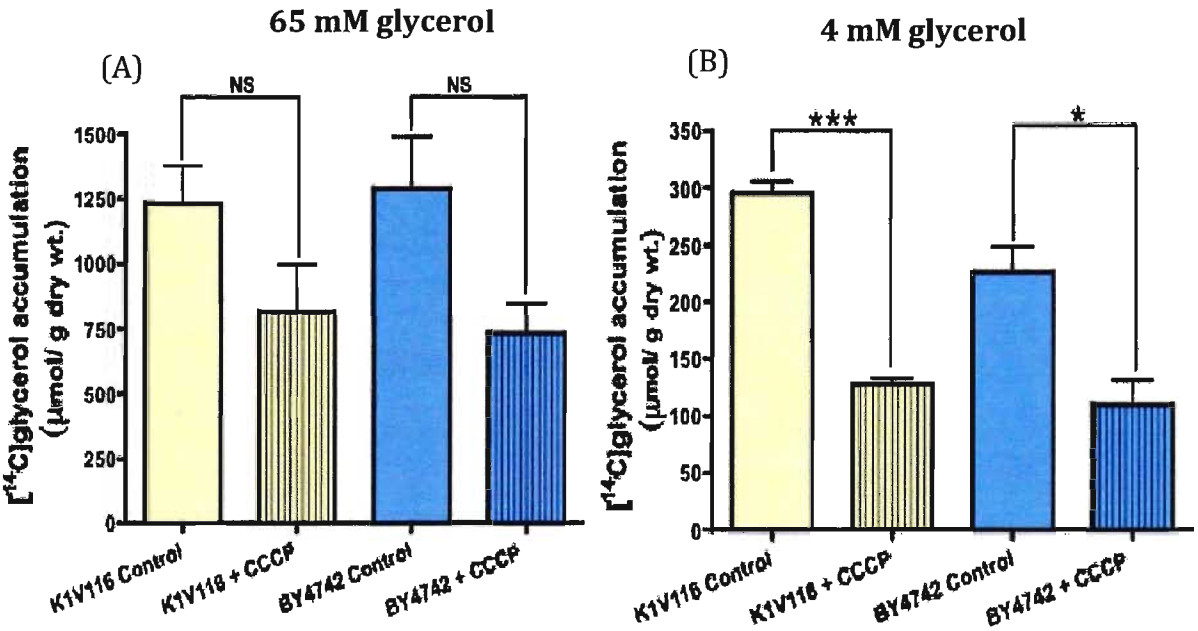

Figure 4.5.1: [^{14}C]glycerol accumulation in wine yeast (K1-V1116) and laboratory strain (BY4742) after 7 minutes with and without the incubation with CCCP at (A) 65 mM and at (B) 4 mM glycerol. *** The difference between the means was extremely significant at the 95% confidence interval as determined by unpaired t-test (n=3, P<0.0001). * The difference between the means was significant at the 95% confidence interval as determined by unpaired t-test (n=3, P=0.018). NS- No significant difference between the means.

At 4 and 65 mM glycerol using Stl1p salt induction conditions, initial uptake rates were not significantly different between parent and wine strains (Figure 4.5.2). It appears that laboratory and wine strain of *S. cerevisiae* behave similarly in relation to glycerol uptake under either higher or lower glycerol concentrations.

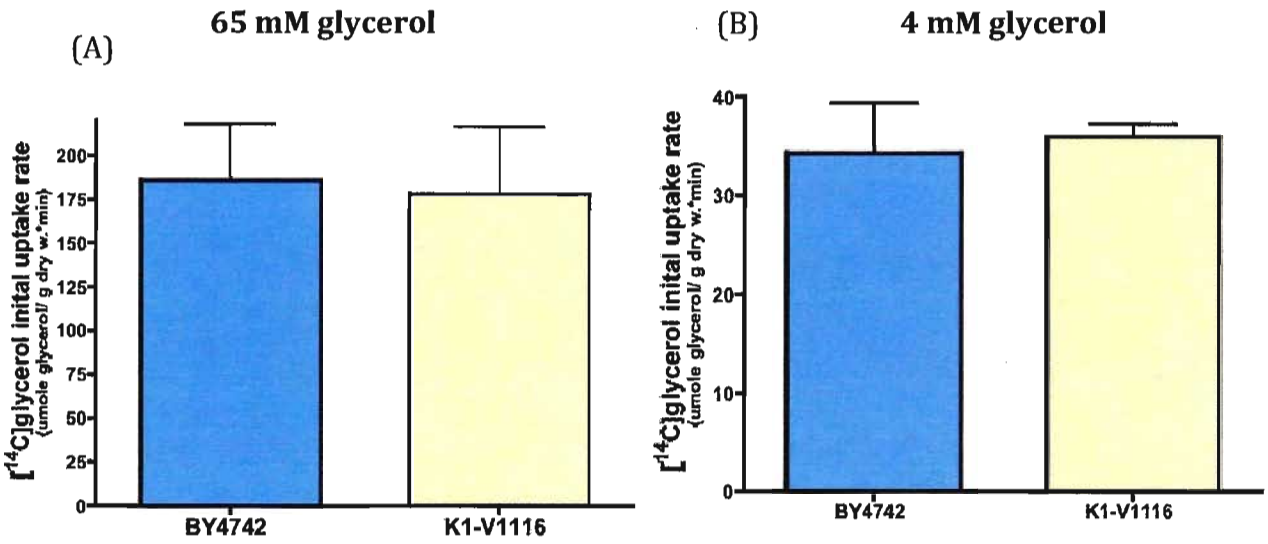

Figure 4.5.2: comparison of [^{14}C]glycerol initial uptake rate in wine yeast (K1-V1116) and laboratory strain (BY4742) at (A) 4 mM and (B) 65 mM glycerol.

To evaluate the contribution of Stl1p in glycerol accumulation in hyperosmotically stressed wine yeast, glycerol accumulation was compared under stressed and non-stressed conditions. Stressed conditions induced glycerol uptake in wine yeast that was correlated to the increase in *STL1* expression upon exposure to the saline stress (Figure 4.1.1).

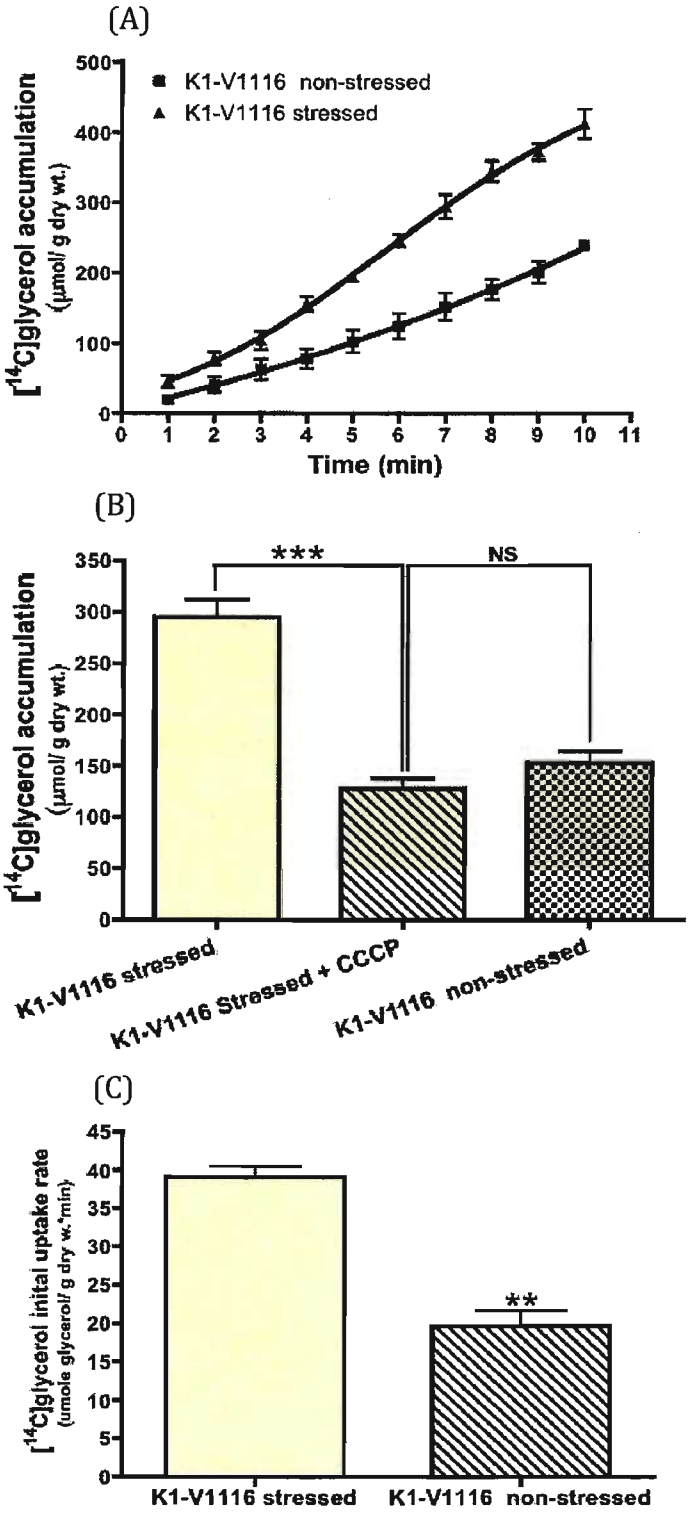

Figure 4.5.3: Comparison of glycerol uptake in stressed and non-stressed wine yeast. **(A)** glycerol uptake **(B)** initial glycerol uptake rate and **(C)** comparison of [^{14}C]glycerol accumulation after 7 minutes in stressed cells with and without the addition of CCCP and non stressed cells. *** The difference between the means was extremely significant at the 95% confidence interval (n=3, P=0.0001). ** The difference between the means was very significant at the 95% confidence interval (n=3, P=0.0015). NS- no significant difference between the means.

Wine yeast cells presented an approximately two-fold increase in the glycerol initial uptake rate and accumulation compared to non-stressed cells (Figure 4.5.3.A and B). Furthermore, the level of glycerol accumulated by non-stressed cells was comparable to the level of glycerol accumulated when stressed cells were incubated with CCCP (Figure 4.5.3.A and B, C). This means that the background level of glycerol entering the cell through mechanisms other than Stl1p constitute approximately half of the glycerol accumulated in stressed wine yeast cells, as observed for laboratory parent strain.

Part 2- Stl1p activity in Icewine and dilute Icewine juice fermentations

To investigate if Stl1p has a role in glycerol uptake in hyperosmotically stressed yeast cells fermenting Icewine, two fermentations were set up (section 3.9-3.10). One with Icewine juice and the second with diluted Icewine juice, containing half of the concentration of soluble solids found in the Icewine juice. The latter fermentation represents conditions at which yeast is exposed to mild hyperosmotic stress and therefore acts as the control condition of table wine fermentation.

The role of Stl1p in wine yeast was investigated first at the RNA level using Northern analysis (section 3.12) and second at the protein level using [^{14}C]glycerol uptake assay (section 3.11).

4.6 STL1 expression in wine yeast fermenting Riesling and Vidal Icewine juices

To investigate the role of Stl1p in hyperosmotically stressed Icewine yeast cells at the gene level, *STL1* expression was compared between cells fermenting Icewine and diluted Icewine (section 3.11). This was done using two different types of Icewine juices, Vidal and Riesling.

Northern analysis of *STL1* expression showed that the gene is not expressed under dilute conditions in wine yeast fermenting either Vidal or Riesling juice, however it is expressed under Icewine conditions (Figure 4.6.1).

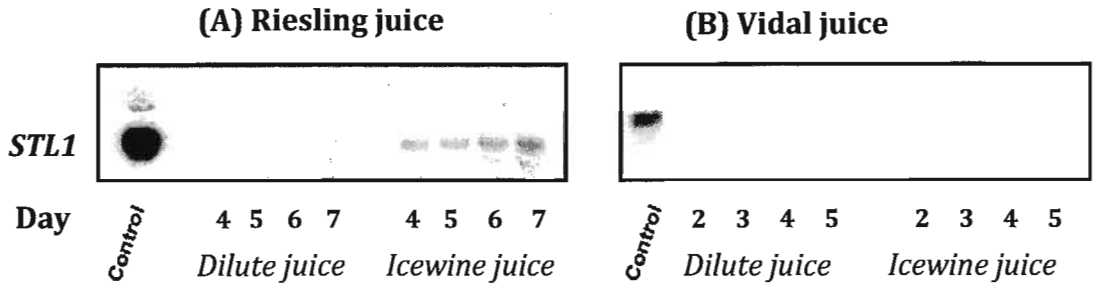

Figure 4.6.1: Expression of *STL1* in wine yeast during Riesling/Vidal Icewine and dilute Icewine fermentations using Northern analysis. RNA of salt stressed BY4742 yeast cells was used as control.

Under Riesling Icewine fermentation *STL1* was expressed in days 4-7, increasing in expression during that period based on signal intensity. Surprisingly, *STL1* was weakly expressed in cells fermenting the Vidal Icewine juice at days 4 and 5 of the fermentation. These results were unexpected since Martin (2008) found that *STL1* was strongly expressed in cells fermenting Vidal Icewine juice by using both Northern analysis and microarray analysis. However, in this experiment a different lot of Vidal Icewine juice was used.

Despite the fact that *STL1* was differentially expressed in this study, the degree of *STL1* differential expression could not be accurately quantified within the timeframe of this thesis study due to technical complications with the breakdown of the phosphoimager machine.

4.7 Stl1p activity in wine yeast during Icewine fermentation

Stl1p activity was compared between cells fermenting Icewine and diluted Icewine that were harvested at day 7 of Riesling fermentation and day 4 of Vidal fermentation (section 3.11).

It appears that glycerol concentration in the fermentation media was correlated to the degree of hyperosmotic stress. Cells fermenting Icewine juice produced significantly greater levels of glycerol compared to dilute Icewine fermenting cells at the day the cells were harvested for glycerol uptake assay (Table 4.7.1). This glycerol was measured in the fermentation media rather than inside the cells, indicating that the cells released the glycerol that was produced in response to hyperosmotic stress.

Interestingly, the level of glycerol concentration measured in Riesling and Vidal Icewine fermentation media was comparable, despite the fact that there was a difference of two days between the fermentations. Also, the concentration of glycerol in the fermentation media was quite similar between the Icewine and the diluted juice media despite the different osmotoic stress the cells were under and their different rates of sugar consumption.

Even though yeast fermenting Riesling Icewine juice were (1) subjected to greater degree of hyperosmotic stress, (2) induced *STL1* and (3) produced higher levels of glycerol at the day the cells were harvested for [^{14}C]glycerol assay, glycerol uptake levels were not significantly different than cells fermenting diluted Icewine juice (Figure 4.7.1). Similarly, no difference in glycerol uptake was detected between

the Vidal Icewine and diluted Icewine fermenting cells, despite the difference in the degree of hyperosmotic stress between the two fermentation conditions.

Table 4.7.1: Glycerol concentration in Riesling and Vidal fermentation media measured on the day the cells were harvested for [^{14}C]glycerol uptake assay

	Vidal day 5		Riesling day 7	
	Icewine	Diluted Icewine	Icewine	Diluted Icewine
Glycerol (g/L)	4.53 ± 0.00	3.96 ± 0.01	4.49 ± 0.00	4.17 ± 0.02
Glycerol produced (g/L)	2.78 ± 0.00	2.86 ± 0.01	2.63 ± 0.07	2.64 ± 0.10
Glucose (g/L)	149.4 ± 1.51	27.3 ± 1.20	173.4 ± 2.8	37.3 ± 1.5
Fructose (g/L)	205.3 ± 6.70	75.7 ± 0.87	241.5 ± 4.07	95.7 ± 2.9
Total sugar consumed (g/L)	31.66 ± 2.60	87.2 ± 1.03	38.89 ± 1.15	89.5 ± 3.78
Glycerol produced/sugar consumed	0.0879 ± 0.005	0.0328 ± 0.006	0.0677 ± 0.001	0.0295 ± 0.004

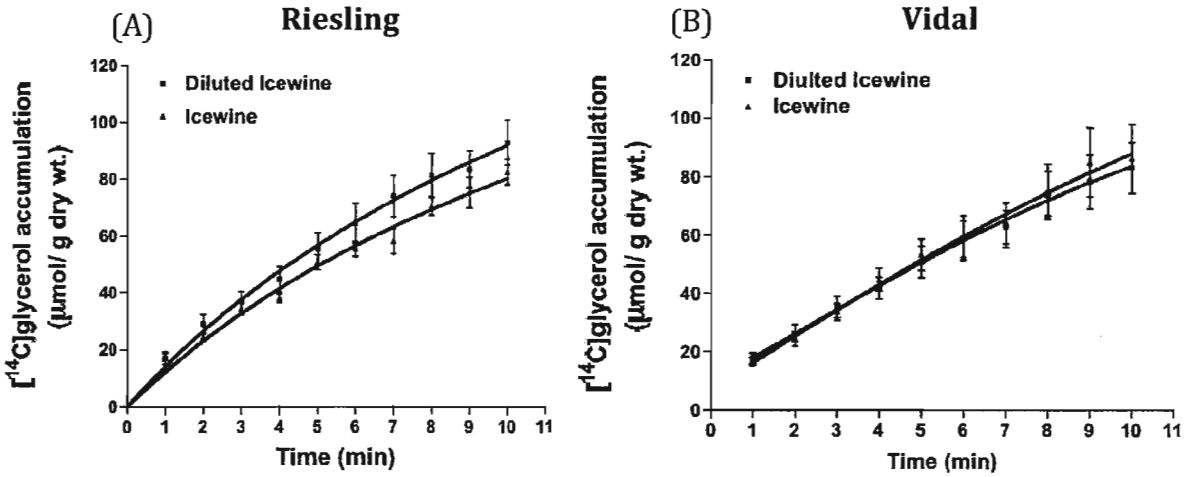

Figure 4.7.1: [^{14}C]glycerol uptake at 4 mM glycerol in wine yeast K1-V1116 fermenting (A) Riesling and (B) Vidal Icewine and diluted Icewine juices.

To better understand whether Stl1p has any contribution to active glycerol uptake during Icewine fermentation, the cells were incubated with CCCP to dissipate

the proton gradient across the plasma membrane and thereby inhibit potential activity of Stl1p (Figure 4.7.2).

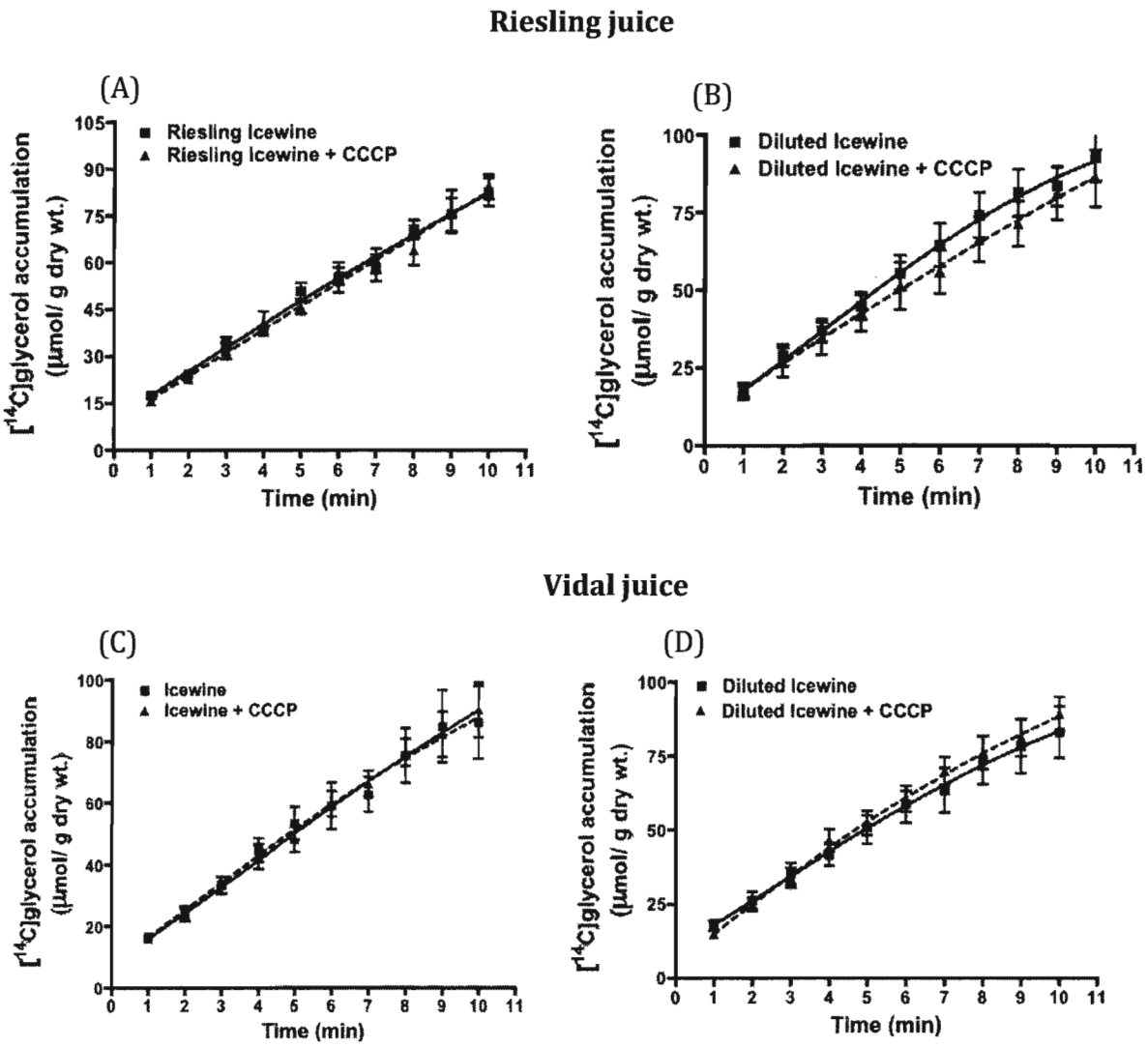

Figure 4.7.2: [^{14}C]glycerol uptake in wine yeast (K1-V1116) during Riesling (A, B) and Vidal (C, D) Icewine and diluted Icewine fermentations in the presence and absence of CCCP.

If Stl1p is present in the Icewine fermenting cells, active glycerol uptake should show sensitivity to CCCP.

Results show that glycerol accumulation in the presence of CCCP was not significantly different than control conditions, where no CCCP was added, under either Icewine or diluted Icewine fermentations for both Riesling and Vidal juices (Figure 4.7.2). These results suggest that wine yeast did not use Stl1p for active glycerol uptake on the day the cells were harvested during Icewine fermentations and Stl1p was perhaps glucose inactivated. It is most likely that this glucose inactivation is not specific for day five of fermentation, however to confirm that, Stl1p activity should be measured throughout the course of the fermentation.

Part 3-*STL1* sequencing

4.8 STL1 sequence alignment

STL1 sequence alignment scored 99% similarity and 99.5% identity between sequences of wine and laboratory strains (Appendix. Figure I). Out of 1710 sequenced base pairs of *STL1* gene (section 3.13) there were 7 codon variations (Table 4.8.2). Four of the codon variations encoded for the same amino acid, therefore, these were silent mutations and the other three variations encoded for an amino acid with the same biochemical properties, hence synonymous mutations.

Substitution in the predicted amino acid sequence occurred in the center of Stl1p within the predicted transmembrane domain (TM) 6 (beta-sheet), between the fifth and the sixth TM (beta-sheet), and between sixth and the seventh TM (α-helix). These mutations did not affect the predicted secondary structures that constitute these domains since they are favored substitution in membrane proteins, according to predicted computational structure analysis.

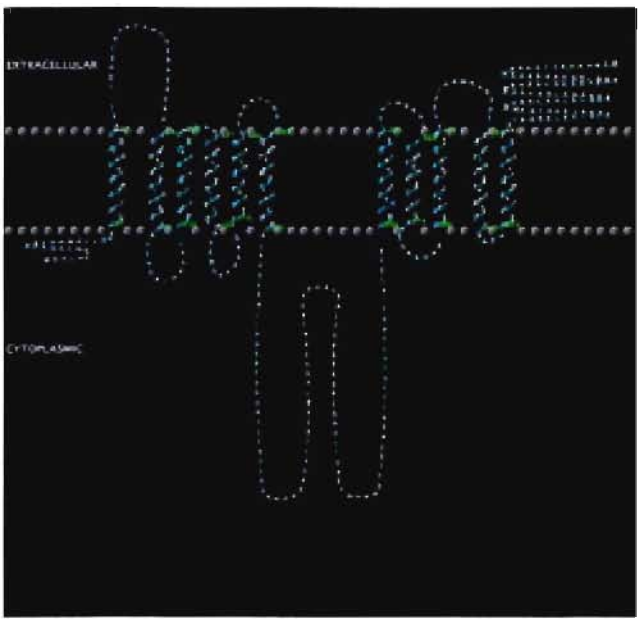

Figure 4.8.1: The predicted 3D structure of Stl1p.

Table 4.8.2: Codon variations in *STL1* sequence of laboratory and wine yeast strains. Grey background represents silent mutations and white background represents synonymous mutations.

Nucleotide position	K1V1116		BY4742		TM Domain (Figure 4.8.1)
	Codon	Amino acid	Codon	Amino acid	
450	TTA	Leu (150)	TTG	Leu (150)	4
546	GGC	Gly (182)	GGT	Gly (182)	5
579	TTT	Phe (193)	TTG	Leu (193)	Between 5 and 6
640	TTC	Phe (214)	CTC	Leu (214)	6
836	AAT	Asn (279)	AGT	Ser (279)	Between 6 and 7
1005	GTG	Val (335)	GTC	Val (335)	8
1296	AAC	Asn (432)	AAT	Asn (432)	11

5 DISCUSSION

The purpose of this study was to investigate the role of Stl1p in glycerol uptake in wine yeast cells during Icewine fermentation. In order to accomplish this goal, first, a glycerol uptake assay dependent on Stl1p had to be developed, and then applied to wine yeast fermenting Icewine juice. After successfully developing this assay, our results show that even though *STL1* was expressed in Icewine fermenting cells, there was no measurable activity of Stl1p using the developed assay on the day the cells were harvested from the Icewine fermentations.

Part 1-Development of Stl1p-dependent glycerol uptake assay

5.1 STL1 is induced in response to saline stress in wine and laboratory yeast strains but not in control strain ΔSTL1

The development of an Stl1p-dependent glycerol uptake assay involved two major steps: first, determination of conditions that stimulated *STL1* expression in the laboratory yeast strain BY4742 where no expression was detected in the negative control *ΔSTL1*; and second, developing an Stl1p activity assay using cells that express *STL1* and using the *ΔSTL1* strain as a negative control to test the specificity of the assay.

In the first step of this project, it was necessary to determine the hyperosmotic stress conditions that resulted in *STL1* induction. This step was important in the development of the Stl1p-dependent glycerol uptake assay to better understand the

relationship between *STL1* expression and Stl1p activity in hyperosmotically stressed cells. To that end, K1-V1116, BY4742 and *ΔSTL1* cells were subjected to 1 M saline stress for the duration of 90 minutes. Results show that under these hyperosmotic stress conditions, K1-V1116 and BY4742 induced *STL1* upon exposure to hyperosmotic stress (Figure 4.1.1) and therefore Stl1p is most likely present in these cells. The absence of *STL1* mRNA signal in *ΔSTL1* cells under either stressed or non-stressed conditions confirmed that this strain did not express *STL1* and therefore, could act as a negative control during the [^{14}C]glycerol uptake assay.

These results also indicated that harvesting the stressed cells at that time point (after 90 minutes of saline stress) should provide the conditions to assay Stl1p activity, since the protein is likely to be present in K1-V1116 and BY4742 however absent in the control strain *ΔSTL1*.

5.2 *The effect of glycerol concentration on Stl1p-dependent active [^{14}C]glycerol uptake measurements*

In the second step of [^{14}C]glycerol uptake assay design, it was necessary to determine the concentration at which glycerol uptake by yeast cells presents both specificity to Stl1p and energy dependency. Yeast cells were exposed to three different glycerol concentrations in the reaction mixture, 4, 10 and 65 mM and glycerol uptake rate and accumulation were compared (Sections 4.2-4.4). In the development of this assay, the commercially available yeast strain *ΔSTL1* developed from the parent strain BY4742 where *STL1* was knocked out of the genome, was

used to assess the background level of glycerol uptake that was not Stl1p-dependent.

Under 4 mM glycerol, *STL1* expression positively contributed to glycerol uptake in hyperosmotically stressed cells. Several pieces of evidence support this conclusion. First, there was a clear difference in the kinetics of glycerol uptake between parent and *ΔSTL1* cells. *ΔSTL1* cells followed monophasic kinetics, where no changes in glycerol uptake rates were detected throughout the course of the assay (Figure 4.2.1). This type of kinetics represents glycerol that entered the cell via passive diffusion (Mattews, 1993; Petty, 1993). Conversely, the parent strain followed biphasic glycerol uptake kinetics, starting with a high initial rate of glycerol uptake that readily declined with time until reaching a steady state rate (Figure 4.2.1). This type of kinetics insinuates a presence of a carrier-mediated glycerol transport, in addition to simple diffusion. Thus, *STL1* deletion eliminated this carrier mediated glycerol uptake observed in parent strain.

Second, deletion of *STL1* significantly reduced the initial rate of glycerol uptake (Figure 4.2.2) suggesting that *STL1* expression is essential to induce rapid glycerol transport into hyperosmotically stressed cells. And third, incubation with the uncoupler CCCP led to approximately 50% reduction in glycerol accumulation (Figure 4.2.3) in the parent strain over the 7 min timecourse experiment, indicating that active glycerol uptake was exclusively inhibited in cells expressing *STL1* and not in *ΔSTL1* cells, thus, the deletion of *STL1* eliminated energy dependent glycerol accumulation.

It is inferred from these results that under the 4 mM glycerol concentration, the expression of *STL1* increases the velocity of glycerol uptake and positively influenced energy dependent glycerol accumulation.

It also appears that at 4 mM glycerol, the glycerol uptake assay can accurately measure Stl1p-depedent active glycerol uptake and accumulation.

The assay was also tested at the higher glycerol concentrations of 10 mM and 65 mM glycerol concentrations that would be encountered by wine yeast fermenting Icewine (Sections 4.3-4.4). These higher glycerol concentrations did not allow accurate measurements of an Stl1p-dependent glycerol uptake as observed by the lack of an energy dependent glycerol uptake in the parent strain and the non-significant difference in glycerol initial uptake rate between the parent strain and its knockout counterpart.

Other than Stl1p, glycerol can enter the cell through facilitated diffusion mediated by the glyceroporin Fps1p, however previously published research showed that under hyperosmotic stress, the Fps1p channel is shut down in order to prevent glycerol loss from the cell and increase glycerol retention (Tamás, et al., 2003). Therefore, the glycerol that was accumulated in *ΔSTL1* cells should not have entered the cells via the Fsp1p channel. Alternatively, glycerol can easily cross the yeast plasma membrane due to its lipophilic nature. That means that the measured experimental velocity of glycerol uptake is encompassing both the contribution of active uptake by Stl1p and passive diffusion.

Oliveira et al (2003) demonstrated that the higher the glycerol concentration used to measure uptake, the higher the contribution of passive diffusion to uptake. This was also observed in our results. Both parental and *ΔSTL1* cells presented higher glycerol uptake rates at 10 mM glycerol compared to 4 mM, and these rates further increased at 65 mM (Figure 4.4.5). In addition, the 65mM high glycerol concentration abolished the biphasic kinetics formerly observed at 4 mM glycerol and eliminated the difference in glycerol uptake rate between parent strain and *ΔSTL1* cells (Figure 4.4.1).

Enhanced passive diffusion not only influenced glycerol uptake rate but also the uncoupling effect of CCCP. *ΔSTL1* did not display sensitivity to the uncoupler at 4 mM, in opposition to the glycerol transport measured at higher glycerol concentrations. These results are in contrast to Oliveira et al (2003) who demonstrated that the CCCP inhibitory effect declines with glycerol concentration. In addition, it is not clear why cells lacking the *STL1* gene would present energy dependent glycerol uptake. These results suggest that Stl1p may not be the only protein responsible for glycerol active uptake in *S. cerevisiae*. Yet, no other genes that code for glycerol symporters have been reported in the literature to date that would suport this hypothesis.

Even though Stl1p may actively transport glycerol into the cell under the 65 mM glycerol conditions, this transport cannot be accurately measured due to the high levels of non Stl1p-dependent uptake of glycerol. This conclusion also holds true for the wine yeast strain tested since it appeared to behave similarly to the parental

strain in relation to glycerol uptake rate and sensitivity to CCCP, under either high or low glycerol concentrations.

Another limiting factor that can influence Stl1p-depenent glycerol uptake is the degree of Stl1p induction upon exposure of the parent cells to hyperosmotic stress. Stl1p induction could be optimized in order to increase the glycerol uptake rate and accumulation in the parent strain, further away from background glycerol uptake. Optimization of Stl1p induction may be advantageous since it can improve the specificity of Stl1p-dependent glycerol uptake at 65 mM glycerol, where background glycerol uptake was shown to be too high, and resulted in non-specific glycerol uptake.

What is the contribution of Stl1p to glycerol accumulation in response to hyperosmotic stress? Salt induction of *STL1* by 3.4 fold in wine yeast led to a two-fold increase in the glycerol uptake rate (Figures 4.1.1 and 4.1.2). This suggests that glycerol uptake rate is *STL1*-dose-dependent. The higher the expression of *STL1,* the greater the effect on glycerol uptake rate in the wine yeast. Interestingly, glycerol accumulation increased by 2-fold as well and this increase was eliminated by CCCP to the same level of glycerol that was accumulated under non-stressed conditions (Figure 4.5.3.B). It appears that the contribution of Stl1p to glycerol accumulation constitute roughly half of the glycerol accumulated in the cell in response to hyperosmotic stress. Therefore, the other half of glycerol accumulated represents glycerol that entered the cell via other transport mechanism such as passive diffusion.

Even though both wine and laboratory yeast strains were exposed to the same conditions of salt stress, *STL1* expression was higher in laboratory yeast strain. However, Stl1p activity was comparable between the two strains. These results further demonstrates that laboratory yeast strain response to hyperosmotic stress differ than wine yeast strain at the level of Stl1p-depenedent glycerol uptake, in addition to the differences that are were previously presented in the gene expression (Borneman et al., 2008).

All these evidence indicate that in wine yeast at 4 mM glycerol (1) glycerol uptake measured in the assay is Stl1p-dependent and (2) glycerol accumulation is energy dependent. On the contrary at 65 mM glycerol, neither Stl1p-dependant uptake nor energy dependent glycerol accumulation could be accurately measured.

Part 2- Stl1p activity in cells fermenting Icewine and dilute Icewine juice

5.3 STL1 was expressed in yeast during Icewine fermentation

STL1 was weakly expressed in cells fermenting Vidal Icewine juice but clearly induced in cells fermenting Riesling Icewine juice over the first 7 days of fermentation (Figure 4.6.1). In the fermentations, day 4 was chosen as the harvest day for the Vidal fermentations and day 7 was chosen for the Riesling fermentations, timepoints where a differential expression of *STL1* was evident at the day the cells were harvested for the [^{14}C]glycerol uptake assay. Surprisingly, a signal for *STL1* expression in was not evident in cells fermenting either Vidal or Riesling diluted Icewine juice. This lack of expression signal for *STL1* in the dilute juice fermentations was surprising based on previous results in Vidal fermentations (Martin, 2008). Martin (2008) demonstrated that *STL1* expression was upregulated in cells fermenting diluted Vidal Icewine during days 2-4 of the fermentation, showing a strong expression on day 4.

More surprising was the weak *STL1* expression during Vidal Icewine fermentation, which once again is in contrast to the findings of Martin (2008) who showed a strong expression of the gene in cells fermenting the same juice type.

It is not clear why cells fermenting Vidal Icewine juice exhibited low *STL1* expression. These results could not have been derived from *STL1* glucose repression where *STL1* transcription is inhibited by glucose, since it was previously shown that *STL1* was expressed despite the presence of high glucose concentration in the juice (Martin, 2008; Erasmus et al, 2003) and *STL1* was expressed in cells fermenting

Riesling Icewine juice. It is probable that this discrepancy resulted from variability among the different Vidal Icewine juice lots. Even though the degree of hyperosmotic stress was comparable to the previous study (Martin, 2008), the chemical composition of the Icewine juice may still differ. The composition of the Icewine juice changes from harvest to harvest depending on different factors such as the environmental conditions the grapes are exposed to, grape ripeness, humidity and more. These factors influence the chemical composition of the grape, and thereby the initial Icewine juice parameters. Vidal Icewine juice parameters that include the initial concentrations of titratable acidity, assimilable amino acid nitrogen, reducing sugars and pH, appear to differ depending on the year of harvest (Kontkanen et al, 2004; Pigeau and Inglis, 2005; Martin, 2008). For instance, the pH of Vidal Icewine juice used by Kontkanen et al (2004) was 0.6 pH units lower than the Vidal Icewine juice used by Martin (2008) and Pigeau and Inglis (2005). It is not known how or if this difference in juice acidity influences the transcriptional regulation of *STL1*.

Alternatively, the low induction of *STL1* during Vidal Icewine fermentation may be a result of experimental error. This potential error could not have resulted from weak binding of the *STL1* probe to the membrane bound mRNA during the hybridization procedure, since the same probe demonstrated a strong hybridization signal with a positive control mRNA (Figure 4.6.1). Furthermore, no visible evidence for experimental error was detected during RNA isolation procedure, besides relatively low RNA yields.

Since Northern blot analysis methods exhibits relatively low sensitivity and requires large amounts of mRNA to accurately quantify gene expression, an alternative method should be used. Real time polymerase chain reaction is a relatively new method that allows rapid and sensitive quantification of gene expression levels (Jürgen & Maurizio, 2005) and requires about 20 times less RNA for a single test than Northern blot hybridization as used in our study. In addition, this method allows a faster quantification of the mRNA fraction, using the internal transcribed spacers *ITS1* as an internal control gene for the normalization procedure.

These advantages will allow enhanced accuracy in RNA quantification when isolation procedure fails to extract satisfactory quantities for Northern analysis.

5.4 Hyperosmotically stressed cells fermenting Icewine did not present active glycerol uptake

Wine yeast fermenting Icewine juice did not show a significant difference in glycerol uptake compared to diluted Icewine juice in either Vidal or Riesling juice, even though Icewine fermenting cells were exposed to greater hyperosmotic stress (Figure 4.7.1). Furthermore, no evidence for active glycerol uptake was detected upon the addition of the uncoupler under either Icewine or diluted Icewine fermentations (Figure 4.7.2).

The glycerol concentration in the Icewine fermentation media was significantly greater than the glycerol concentration found in the diluted Icewine fermentation as observed in previous studies (Martin, 2008; Pigeau and Inglis, 2005).

Furthermore, Icewine fermenting cells produced more than twice of the glycerol produced by diluted Icewine fermenting cells, for the same amount of sugar consumed for both juice types (Table 4.7.1) as previously observed by Martin (2008) and Pigeau and Inglis (2005). It was hypothesized in this project that Stl1p has a role in the uptake of the glycerol that was initially present in the fermentation media and subsequently released during fermentation, to increase the level of intracellular glycerol and counteract the dehydration effect of the Icewine juice. However, hyperosmotically stressed cells harvested from Vidal and Riesling Icewine fermentations on day 4 and 7 respectively did not show energy dependent glycerol uptake activity that differed between cells in the Icewine or diluted Icewine conditions (Figure 4.7.2.A and C).

Even more surprising was the absence of active glycerol uptake specifically in the cells fermenting Riesling Icewine juice, since these cells induced *STL1* at the day of their harvest (Figure 4.6.1.A).

Why did hyperosmotically stressed cells not show an energy depended glycerol uptake however still expressed *STL1* during Riesling Icewine fermentation?
The fact that active glycerol uptake was not detected in hyperosmotically stressed wine yeast cells despite the increase in glycerol concentration in the fermentation media suggests that Stl1p was probably glucose inactivated in wine yeast. These

results are in contrast to the findings of Ferreira et al (2005), who showed that Stl1p glucose inactivation was overcome by saline hyperosmotic stress in laboratory yeast cells grown on glucose based media (Ferreria, et al., 2005).

These results may imply that overcoming Stl1p glucose inactivation in *S. cerevisiae* depends on the type of stressor used to induce the hyperosmotic stress. Salt induces Stl1p active glycerol uptake whereas the high concentration of soluble solids in the Icewine juice most likely leads to Stl1p glucose inactivation and subsequent inhibition of active glycerol uptake.

In addition to the type of stressor, overcoming Stl1p glucose inactivation may also depend on the yeast strains. Studies have shown that response of laboratory yeast strain to hyperosmotic stress differs from commercial yeast strain at the transcriptional level (Rep, et al, 2000; Pigeau and Inglis, 2005, Erasmus et al, 2003; Martin, 2008). In addition, Kayingo et al (2009) demonstrated that unlike glucose-inactivated Stl1p of laboratory strain of *S. cerevisiae* under non-stressed conditions, the activity of *C. albicans* glycerol symporter is unaffected by carbon source (Kayingo, et al. 2009). Borneman at al. (2008) demomstrated that 0.6% of the whole genome and 0.4% of predicted proteome differ between the laboratory and wine yeast strains. These genomic variations may lead to Stl1p glucose inactivation in wine strain, but not in laboratory strain during hyperosmotic stress.

Therefore, it is likely that overcoming Stl1p glucose inactivation by hyperosmotic stress may be a strain-dependent event in addition to the type of solute used to stress the cells.

The absence of energy dependent glycerol uptake measured in cells fermenting Icewine could also have resulted from low sensitivity of the designed [^{14}C]glycerol assay. The sensitivity of glycerol uptake assay was initially fixed to allow measurements of energy dependent glycerol uptake of salt stressed wine yeast grown on ethanol-based media. Taking into account the degree of *STL1* expression in wine yeast under these conditions, it was determined that the designed assay showed competency to measure Stl1p active glycerol uptake. However, this degree of sensitivity was not sufficient to measure glycerol uptake in Icewine and diluted Icewine fermenting cells. For that reason, the cell concentration in the reaction mixture had to be increased by 1.5-fold in order to increase the assay sensitivity. Still, the maximum level of glycerol accumulation in cells fermenting Icewine juice (Figure 4.7.1) was four times lower than the level of glycerol accumulated in wine yeast grown on ethanol and stressed with salt (Figure 4.5.3.A). It appears that the designed assay may present sensitivity limitations for measuring Stl1p in wine yeast during fermentation and therefore may not be suitable in its present form to detect differences in glycerol uptake between cells that were harvested from Icewine and diluted Icewine fermentations.

Part 3-STL1 sequencing

5.5 Glucose inactivation and Stl1p activity in wine and laboratory yeast strains

Our results suggest that overcoming Stl1p glucose inactivation by yeast under hyperosmotic stress conditions is either solute dependent or strain-dependent. Therefore, a third objective was set to compare the coding sequence of *STL1* between laboratory and wine yeast strains of *S. cerevisiae* in order to evaluate for potential variability in the predicted amino acid sequence and consequently the predicted 3D structure of Stl1p of the two strains. These differences may lead to changes in the tertiary structure of Stl1p and thereby affect the process of glucose inactivation.

STL1 coding sequence alignment of wine and laboratory yeast strains revealed that *STL1* coding sequence was 99.5% identical between the two strains. The seven-codon variations resulted in four silent and three synonymous mutations (Figure 4.8.1). Since silent mutations do not result in amino acid substitution, these mutations cannot influence the secondary or tertiary structure of Stl1p. On the other hand, the synonymous mutations may affect the tertiary structure of Stl1p, even though these are favorable amino acid substitutions for membrane proteins. It is difficult to predict this effect without the crystal structure of Stl1p, which has yet to be investigated.

Stl1p glucose inactivation occurs through the ubiquitination of Stl1p. The addition of this short peptide targets Stl1p for degradation in the vacuole. However,

the chemical nature of the signal targeting Stl1p glucose-induced degradation is unknown.

Glucose inactivation of fructose-1,6-bisphosphate (FBPase) has been extensively studied. The enzyme is first reversibly inactivated by phosphorylation on a serine residue and then irreversibly inactivated by proteolysis (Müller and Holzer 1981). Mutations of Serine 289 resulted in the decrease of glucose induced FBPase inactivation (Hung et al. 2004). Interestingly our results suggest that the expected amino acid sequence of Stl1p wine and laboratory yeast strain differs at the 279 position located between transmembranes domains six and seven. This substitution of serine to aspargine in wine yeast strain can possibly influence the inactivation of Stl1p in the presence of glucose, since this amino acid residue appears to be important for the phosphorylation of proteins during glucose inactivation event. If this is true, this substitution may eliminate Stl1p glucose inactivation in wine yeast. However, this assumption does not agree with our conclusions that Stl1p was most likely glucose inactivated in wine yeast during Icewine fermentation.

6 FUTURE DIRECTIONS

6.1 Optimization of Stl1p induction

The designed glycerol uptake assay could measure Stl1p activity at 4 mM glycerol, however, failed to do so at higher glycerol concentration such as 65 mM. Even though Stl1p may actively transport glycerol into the cell at 65 mM glycerol, this transport cannot be accurately measured due to the high levels of background glycerol uptake that represent the non Stl1p-dependent uptake of glycerol.

In order to reduce the effect of background glycerol uptake at 65 mM glycerol, Stl1p-dependent glycerol uptake should be optimized in parent strain (BY4742). Greater induction of Stl1p may increase the glycerol uptake in parent strain above background levels.

In this study, the expression of *STL1* was measured only at one timepoint, after 90 minutes of 1 M NaCl hyperosmotic stress. However, it is not known at which time cells present the peak expression of *STL1*, and how this expression correlates the induction of Stl1p. Therefore correlating the degree of *STL1* expression and the activity of Stl1p will create a dose-response curve. To that end, *STL1* expression should be measured at 30, 60, 90 and 120 minutes upon exposure to 1 M NaCl hyperosmotic stress, and at the same timepoints, the initial glycerol uptake rate should also be measured to in laboratory strain. The relationship between the degree of *STL1* expression and Stl1p-dependent glycerol uptake will present the dose-response curve of Stl1p. This curve will allow to determine (1) if glycerol

uptake in parent strain cells depends on the concentration of Stl1p in the cell membrane, and (2) the timepoint at which Stl1p induction peaks throughout the time course of hyperosmotic stress. Harvesting the cells at the timepoint of Stl1p peak activity will potentially increase the levels of glycerol uptake, above background glycerol uptake, at 65 mM glycerol.

6.2 Further investigation of wine yeast Stl1p glucose inactivation

The fact that active glycerol uptake was not detected in hyperosmotically stressed wine yeast cells despite the high concentration of soluble solids in the Icewine juice suggests that Stl1p was glucose inactivated in K1-V1116 strain cells. Because Icewine juice is a complex media that comprises different metabolites and chemicals, it is important to make certain that the absence of active glycerol uptake in Icewine fermenting cells directly resulted from Stl1p glucose inactivation, rather than inhibition by the components in the Icewine juice.

To that end, it is necessary to provide further supporting evidence for Stl1p glucose inactivation in wine yeast harvested from Icewine fermentation. In order to confirm that glucose is the component in the Icewine juice that is responsible for the inhibition of energy dependent glycerol uptake in wine yeast cells fermenting Icewine juice, the activity of Stl1p should be explored in wine yeast cells in the presence and absence of glucose. Stl1p activity will be measured in cells suspended in a buffer media containing glucose concentrations that are normally found in Icewine juice (200 g/L).

To show that Stl1p in wine yeast is glucose inactivated as previously demonstrated in laboratory yeast strain (Ferreira, et al, 2005), Stl1p-dependent glycerol uptake assay used in this project will be adjusted to measure Stl1p activity in the presence of glucose.

In the first step, the inhibition of active glycerol uptake in parent strain due to Stl1p glucose inactivation will be validated in laboratory strain of *S. cerevisiae*, and therefore will act as control for Stl1p inhibition in wine yeast. K1-V1116, BY4742 and *ΔSTL1* cells will be grown on ethanol-based media and stressed with 1 M NaCl to induce *STL1*. The induction of *STL1* in parent and wine yeast strains and the lack of *STL1* expression in the control strain *ΔSTL1* will be confirmed using Northern analysis, as it was done for this project.

The cells will then be harvested for [^{14}C]glycerol uptake assay to measure Stl1p-dependent active glycerol uptake using the same *STL1* induction conditions. Following that, cells harvested from the same experiment will be suspended in a buffer solution containing 2 g/L of glucose (11 mM) for 30 minutes. Ferreira et al. (2005) demonstrated that when laboratory strain cells grown on non-fermentative carbon source were shifted to media containing 2 g/L of glucose, Stl1p was completely glucose inactivated after 30 minutes of exposure to glucose, however it is not known if Stl1p is glucose inactivated in wine yeast under the same conditions.

After validating that *STL1* was induced and Stl1p activity is present in ethanol grown laboratory yeast cells, Stl1p-dependent glycerol uptake will be measured once again, following the incubation with glucose and compared between the two

conditions. If the difference in the initial glycerol uptake rate between parent strain and *ΔSTL1* is eliminated in the presence of glucose, it is possible to infer that active glycerol uptake was inhibited in laboratory yeast strain due to glucose addition, as observed by Ferreria et al (2005). To test if this glycerol uptake is an energy dependent process, the % reduction in glycerol accumulation will be compared before and after the addition of glucose, in the presence and absence of CCCP. If parent strain cells incubated with glucose does not present a significant difference in glycerol reduction upon incubation with CCCP compared to *ΔSTL1* cells, it is possible to conclude that active glycerol uptake is inhibited in parent strain cells due to Stl1p glucose inactivation. The same procedure will be used to test Stl1p glucose inactivation for laboratory yeast cells suspended in 200 g/L of glucose, a concentration of glucose that is often present in the Icewine juice.

Upon validation of Stl1p-glucose inactivation using the control assay with laboratory yeast strain, the assay will be applied to measure Stl1p activity in wine yeast strain in the presence of glucose.

Using the same procedure as determined for the control assay, the initial glycerol uptake rate will be compared before and after incubation with glucose. To test for active glycerol uptake, glycerol accumulation will be compared in the presence and absence of CCCP with and without the 30 minutes incaution with 2 g/L of glucose. Reduction in the initial rate of glycerol uptake upon exposing the cells to glucose will indicate that glycerol uptake is partially inhibited in the presence of glucose. If the level of glycerol accumulation in wine cells will not be affected by the

addition of CCCP in the presence of glucose, it will indicate that active glycerol uptake was inhibited due to Stl1p glucose inactivation.

Salt-induced hyperosmotic stress was previously shown to overcome glucose inactivation in laboratory yeast strain, however it is not known if sugar-induced hyperosmotic stress results in the same outcome. Therefore, in the last step, Stl1p-glucose inactivation will be evaluated in wine yeast using the same approach except the cells will be exposed to 200 g/L glucose solution (1.1 M), a concentration of glucose that is often encountered by yeast during Icewine fermentation. If Stl1p glucose inactivation can overcome by glucose-induced hyperosmotic stress, the glycerol initial uptake rate will not change upon exposure to glucose stress, and the cells should experience sensitivity to CCCP, indicating of energy dependent glycerol transport.

7 CONCLUSIONS

We concluded that active glycerol uptake is not detected in wine yeast during Icewine fermentation, most likely due to Stl1p glucose inactivation, Therefore, Stl1p cannot contribute to the dissipation of the proton gradient and the limited cell growth observed during the process of Icewine fermentation.

8 LITERATURE CITED

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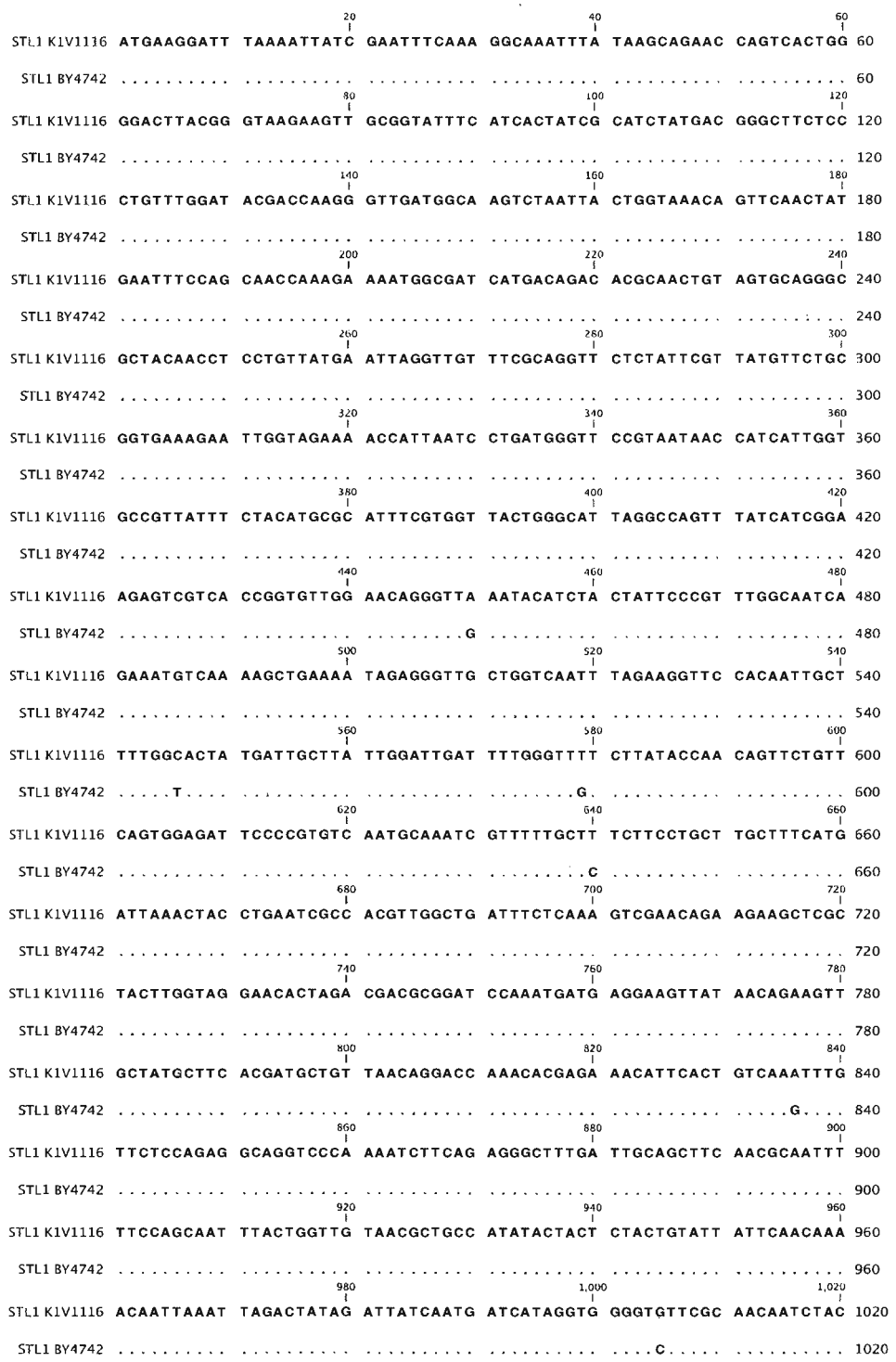
9 APPENDIX

9.1 STL1 sequence alignment



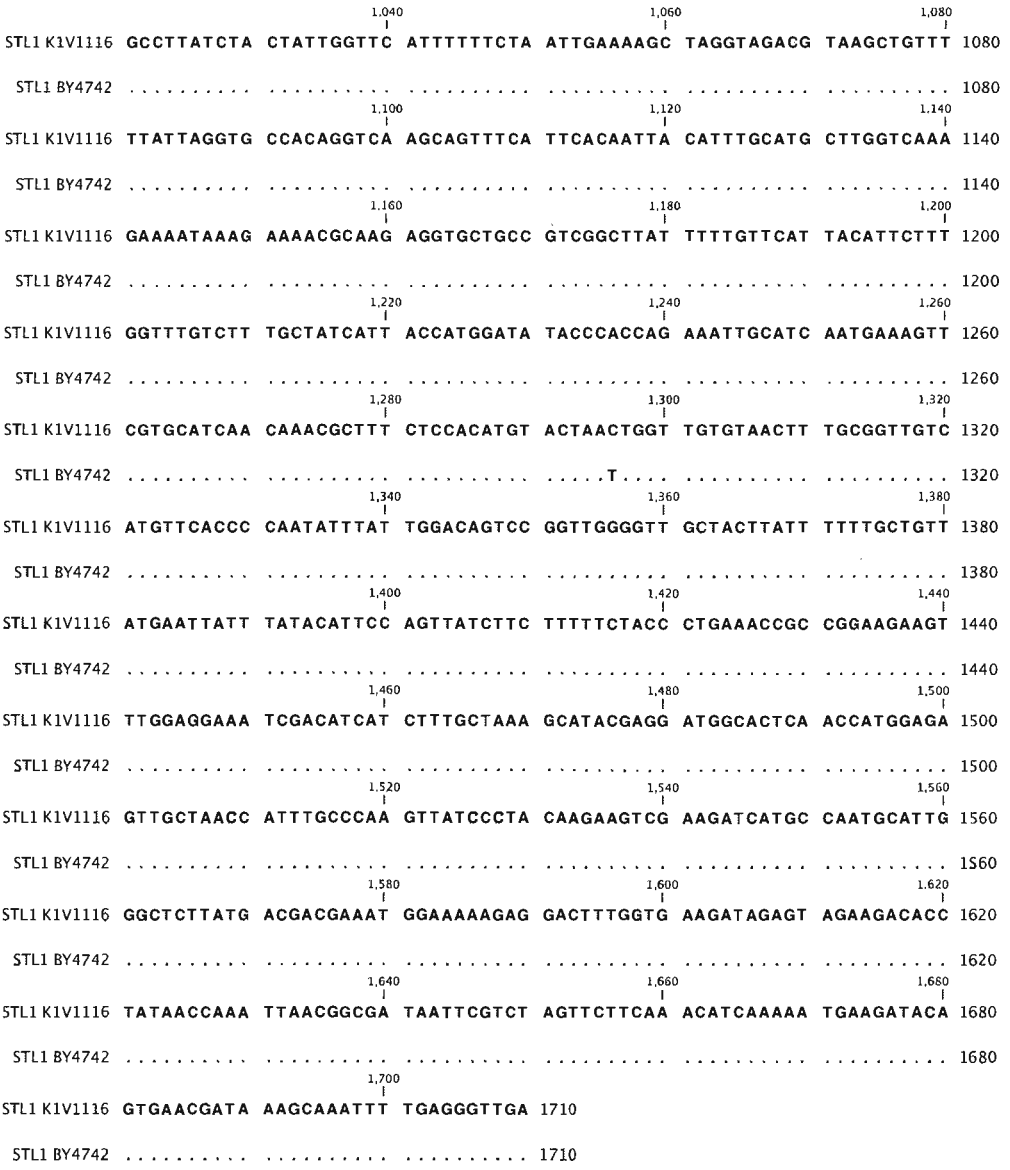


Figure 9.1.1: Sequence alignment of K1-V1116 and BY4742 STL1 gene. Figure created using CLC main Workbench V 4.2.0.

9.2 Optimization of glycerol uptake assay

In the designed glycerol uptake assay, hyperosmotically stressed cell were incubated with [^{14}C]glycerol and 10 uL of stock cell suspension was filtered at specific time points and washed with Ice-cold water to remove excess extracellular glycerol and allow accurate measurements of the level of intracellular [^{14}C]glycerol. The level of glycerol accumulated by the cells was inferred from the amount of radioactivity that was left on the filter. Because glycerol has a lipophilic nature there was a concern that the radioactivity that remained on the filter represents extracellular glycerol that was bound to the cell membrane rather than intracellular glycerol. That means that washing the cells with water may not be sufficient to remove extracellular glycerol and therefore hinder the accuracy of the results.

To make certain that the radioactivity retained on the filter represents only intracellular glycerol, cells were washed with either Ice-cold water or 1 M cold glycerol solution. If radiolabeled glycerol was indeed stuck in the cell membrane, washing the cells with cold glycerol should reduce the radioactivity on the filter since the cold glycerol molecules will compete and therefore substitute for the membrane-bound radiolabeled glycerol.

Washing *ΔSTL1* or parent strain cells with glycerol did not decrease the level of radioactive glycerol throughout the course of the assay (Figure 4.2), indicating that glycerol was not bound to the cell membrane and therefore washing with water is sufficient to remove extracellular glycerol from the filter.

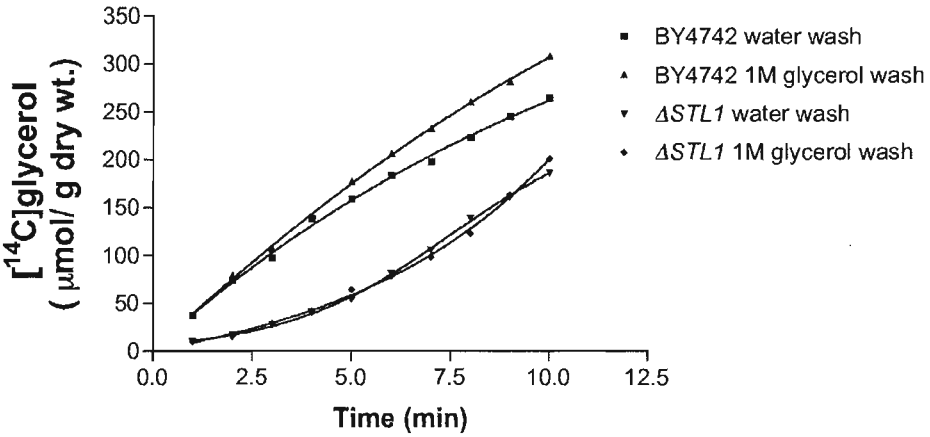


Figure 9.2.1: The effect of washing solution on the level of radiolabeled glycerol retained on the filter. Parent strain and *ΔSTL1* cells were washed twice with either 5 mL of Ice-cold water or 5 mL of cold glycerol solution (1M) at the specified time-points.